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13. ABSTRACT (Maximum 200 Words)

This project studied the genetic basis of tumor pathogenesis in neurofibromatosis 1 (NF1). This work tested the two-hit hypothesis in NF1 tumors (neurofibromas and MPNSTs), and involvement of other loci such as TP53. Human tumor samples were collected, with development of cell cultures from some. Discoveries: the two-hit mechanism operates in at least a large proportion of neurofibromas (which may include methylation as a mechanism); there is a genetically abnormal clonal Schwann cell component in these tumors; somatic isodisomy may be present in loss of heterozygosity cases; the TP53 gene (and RB1 and P73) is implicated only in MPNSTs; and the ErbB2 gene may be a modifier of NF1. Also, the NF2 gene has allelic loss in some MPNSTs and plexiform neurofibromas. An antisense NF1 series of experiments showed only 15% reduction in NF1 mRNA with the best construct, too low to adequately test the tumorigenesis theory given that we found absence of neurofibromin on Western blot analysis of human tumor-derived Schwann cell cultures. Data from an expression array study implicated 9 genes in plexiform tumorigenesis (involved in apoptosis and extracellular matrix). Collectively, these data will contribute to development of NF1 tumor therapies.

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INTRODUCTION

Neurofibromatosis type 1 (NF1) is a frequent autosomal dominant disease that displays variable expressivity, and is due to a mutation in the NF1 gene on chromosome 17. The features involve localized overgrowths or abnormal growths of neural crest-derived tissues. Thus, NF1 is characterized by abnormal cell proliferation, particularly evident in the formation of benign neurofibromas and malignant peripheral nerve sheath tumors (MPNSTs). Neurofibromas contain mostly Schwann cells, which are thought to be a key cell type in these tumors. Nearly all NF1 patients develop dermal neurofibromas (small tumors in or under the skin on peripheral nerve twigs), 20-40% of patients develop plexiform tumors (larger, typically arising on deeper nerves), and about 5% develop MPNSTs (these usually occur within a previously-existing neurofibroma). Presently there are no known measures for the prevention of NF1 tumor development, and treatment of these tumors, which involves use of standard therapies has not proven particularly effective because this is a lifelong progressive disorder and complete removal of the tumor sacrifices the attached nerve. Understanding of the progression of normal cells to a benign tumor and ultimately to malignancy is lacking in NF1; such knowledge would be invaluable for the development of preventive strategies, diagnostic tools and therapeutic approaches. Our work involves investigating the two-hit hypothesis in neurofibromas, which is the theory that the originating cell of a tumor contains the inherited/germline NF1 gene mutation plus a somatic mutation on the remaining allele, resulting in functional inactivation of NF1 and its protein. Due to the large size of the NF1 gene (60 exons, encoding 2818-amino acid protein), and the wide variety of NF1 mutations, detection of both germline and somatic NF1 mutations is challenging. A variety of genetic methods are being used to search for abnormalities in NF1, TP53, and other tumor-related genes, as well as screen for genetic and/or regulatory changes that might involve yet-unidentified genes, through several more global genetic analyses. Another aim is to study the functional effect(s) of NF1 and TP53 inactivation in Schwann cells through the use of antisense inhibition technology, as an alternative method for testing the two-hit hypothesis. The material for this work consist of solid tissue and cell cultures of NF1 tumors contributed by patients, which has provided and excellent resource for these and collaborative investigations. The data generated from these experiments will lead to a more comprehensive understanding of the pathogenesis of tumors in NF1, to provide targets for future therapies.

Technical Objective A. Establish that NF1 Tumors Conform to the "Two-Hit" Hypothesis for the NF1 Gene.

Progress on Task A1: Continue/complete preparation of samples (DNA and RNA) from the current solid tissue and cultures. Continue subculture of tumoral Schwann cells.

Completed. We have prepared RNA and DNA from all of the solid tumors and cultures established to this point. In this last year we received tumor and blood samples from 3 NF1 patients, to add to the ongoing experiments. Nucleic acids were extracted, and Schwann cell enrichment through cell culture was accomplished for all 3 tumors (1 dermal neurofibroma, 1 plexiform neurofibroma, and one MPNST). Schwann cells from 2 normal (non-NF1) nerves were also cultured for additional controls. We included these new samples in the analyses that were being finished in this no-cost extension year. During the period of this grant (Oct '98 to current) we obtained 32 dermal tumors (establishing enriched Schwann cultures on 5), 19 plexiform tumors (cultures on 8), and 2 MPNSTs (culture established), which we added to the samples previously obtained in NIH-funded work. The description of establishing the neurofibroma cultures and lack of neurofibromin in some of these cultures (immunostaining) was published in 2001 (Muir et al., 2001, see Appendix). We are also sharing these resources with other investigators (including some DOD-NF program funded investigators) such as Dr. Lynn Fieber (Univ. of Miami), Dr. Karen Stephens (Univ. of Washington), Dr. Yi Zhong (Cold Spring Harbor), and Dr. Nancy Ratner (Univ. of Cincinnati). Some collaborations have resulted in data being considered for publication.

Progress on Task A2 : Complete intragenic loss of heterozygosity (LOH) studies on all available tumors; complete studies to outline extent of NF1 deletions.

Completed. LOH analyses have been extended throughout the project to include new tumor samples and culture DNAs. Our work through 1999 was published in 2000 (Rasmussen et al., 2000, see Appendix) in which 5% of dermal, 40% of plexiform neurofibromas (solid tissue only), and 60% of MPNST samples showed LOH in the NF1 gene region. We now have 14 NF1 intragenic markers in the lab (7 restriction fragment length polymorphisms (RFLPs), 7 microsatellites), as well as some RFLP and microsatellite markers across the p and q arms to help delineate regions of LOH. Current data, up to date for most markers in all samples in the lab, shows NF1 LOH in 18/71 (25%) dermal neurofibromas, 19/53 (36%) plexiform tumors, and 6/7 MPNSTs (solid tissue, except 3 MPNSTs exist as culture). The percentage of LOH is slightly less for the Schwann-enriched cultures, but the purity of LOH tends to be greater in these and is consistent with LOH in primary tissue. This supports the notion that the cultures are enriched for tumorigenic Schwann cells and that there is a clonal population of somatically mutated Schwann cells, consistent with the 2-hit hypothesis. The data continue to be consistent in indicating that LOH in both types of neurofibromas does not extend proximally beyond the marker HHH202, but may extend distally through the most distal marker we have on the q arm, in the last cytogenetic band. Some LOH regions are limited to within the NF1 gene. The MPNSTs, in contrast, have LOH data that suggest loss of a complete homolog, although one sample shows retention of the single most distal marker on 17p and 17q, suggesting a complex rearrangement (but with most of 17 being deleted, including NF1 and TP53, the latter of which is on 17p). Newer LOH data will be included in a paper in preparation that will describe tumors for which both the germline and somatic NF1 mutations are reported (Thomson et al., in preparation), and in Ms. Thomson's Ph.D. dissertation (in preparation).

An offshoot of this project was a Southern blot analysis of LOH-positive samples, with consecutive probing of the blot with a dizygous control probe and a polymorphic NF1 probe. The goal, along the lines of understanding 2nd-hit mechanisms in NF1, was to

determine whether the allele loss was truly a deletion (hemizygosity) or a deletion with replacement of the sequences from the germline-mutant chromosome (isodisomy). The first analysis, last year, studied three tumors with strong LOH, two with weak LOH, and several with no obvious LOH (as well as a germline normal and germline large deletion cases, with known heterozygotes and homozygotes). The control probe was a non-polymorphic sequence from chromosome 15 as a source of normalization factor (DN34), and the polymorphic probe within NF1 was EVI2B (producing an EcoRI RFLP, thus EcoRI was used for the Southern blot digest). Data were gathered on a phosphorimager, and the intensities clearly support the notion that the remaining allele (containing the germline mutation) is present in 2 copies in the strong-LOH tumors (which were all plexiforms). The weak LOH tumors also yielded numbers for the germline copy that were greater than hemizygous, but not as strong (not clearly dizygous, but in that direction) (probably related to amount of contaminating normal tissue in the tumor; these were dermal tumors). These data suggest that isodisomy is acting in benign neurofibromas, although we know from previous data mapping range of LOH that the isodisomic region must vary, in contrast to leukemias. This is consistent with data published recently by another group using cytogenetic tools (Serra et al., 2001). This year a new Southern blot with 2 additional LOH-positive samples was analyzed, and preliminary results also indicate isodisomy, although we are not satisfied with the control hybridization signal and plan to repeat this so it can be published. Isodisomy would be an explanation for the lack of cytogenetic aberrations (at least on chrom. 17) in tumors that show LOH. This finding is of importance in understanding neurofibroma pathogenesis since there may be a significant biochemical difference between a tumor hemizygous for a large region versus one that is dizygous for the same allele of the same genes.

Progress on Task A3: Complete NF1 protein truncation test (PTT) studies on all tumors for which NF1 LOH was not found to identify somatic mutations; complete NF1 PTT on all blood samples to identify germline mutations; characterize specific mutations (germline and somatic) on cases in which PTT has identified truncated proteins.

Essentially complete. As described in previous progress reports, many solid tumor RNAs were found to be of insufficient quality to yield unambiguous PTT results. Six tumor RNAs showed positive PTT results, with 3 reflecting the germline mutation and 3 being the somatic mutation. Another round of PTT analysis is now finishing, including RNAs from a few Schwann or fibroblast cultures grown with puromycin to enrich for transcripts containing truncating mutations, by inhibiting nonsense-mediated decay (Messiaen et al., 1999 (a collaborative publication, see Appendix), 2000).

It now appears that under standard conditions, the PTT is also not an optimal test, due to the discovery of aberrant splicing in the NF1 gene due to sample handling. This was originally discovered by us (Wallace et al., 1998 abstract; Messiaen et al., 1999), but was first published in manuscript form by several other groups (e.g. Wimmer et al., 2000). We have characterized these forms, which are consistently missing partial or whole exons. These are related to exposure of blood to room temperature conditions for at least an hour; this is avoided with immediate processing or immediate refrigeration; this effect is minimized with blood tubes meant to stabilize RNA. Our results were recently published (Thomson et al., 2002a, see Appendix). The paper also reports a screen of multiple tissues for alternative NF1 exons and did not find any new substantial forms other than those previously reported, although there appear to be minor differences for some isoforms that are in quite low quantity.

Because of the above pitfalls discovered with the PTT, supplementary SSCP and CpG nonsense screening analysis of the most frequently-mutated exons and recurrent mutations (1, 4a, 4b, 7, 10a, 10b, 11, 13, 16, 20, 21, 22, 23.2, 24, 25, 29, 31, 33, 37, 41, 42, 44, 45, 49 (300 bp spanning stop codon)) was also applied to help find germline and somatic mutations in tumors. 68 tumor DNAs were mostly from plexiform tumors and tumor cultures (non-LOH), and in this set of DNAs in these exons, 17 mutations have been characterized, along with detection of 4 rare neutral variants. In the lab, including work prior to this grant and samples obtained during this time, a total of 70 germline NF1 mutations have been identified in our patients (some of whom have contributed tumors to this study), and 7 specific somatic NF1 mutations (not LOH). Of these latter 7, 6 are clearly inactivating (3 nonsense, 3 frameshift) although one is a splicing error that causes a skip of exon 10a. This would encode a protein lacking 44 amino acids but otherwise normal, which may point to important domains in exon 10a (which is upstream of the GAP domain). Some of these data contributed to a recently published NF1 mutation review-type paper (Thomson et al., 2002b, see Appendix), and will also contribute to the Thomson et al. two-hit paper in preparation mentioned above. Of the 70 germline mutations, 88% are clearly predicted to produce a substantially altered protein (truncated or missing many residues, or completely deleted). Of the 12% "missense" mutations, most have been evaluated at the RNA level and most do not have an apparent effect on splicing, 3 remain to be tested and it would not be surprising if one or more actually alter splicing, increasing the percentage of "severe" mutations. A joint publication with Dr. Messiaen (Belgium) illustrated how a coding region point change can affect splicing, and illustrated a recurrent mutation in exon 10b (Messiaen et al., 1999; Appendix). One particularly interesting germline mutation found in a patient with a heavy dermal neurofibroma burden is a novel exon 15 missense mutation which does not affect splicing, and thus represents a true amino acid substitution, in a conserved residue outside the GAP domain. Such mutations are of interest in helping define functional motifs of neurofibromin, and may suggest that alternative pathogenetic mechanisms occur in some situations (other than truncated protein). We also discovered a 1 bp deletion in the 3′ UTR (2.2 kb beyond the stop codon) in one patient that is novel, which may lead to future experiments testing for pathogenetic effects (this would be the first disease-causing 3′ UTR NF1 mutation).

Collaborator Dr. Karen Stephens (Univ. of Washington) tested over 100 of our tumor DNAs for the large repeat-mediated gene deletion in tumors (using their PCR-based assay, Lopez-Correa et al. (2001)) but found no somatic deletions. This suggests that this mechanism is not important mitotically as a contributor to neurofibroma pathogenesis. Her lab also screened our known and suspected large germline deletion patients to characterize the type of deletion based on which flanking repeats were used to mediate the deletion, and found 2 new germline deletions in patients that we had previously found homozygous at all NF1 markers (Dr. Stephens is preparing a manuscript, we are co-authors).

Also, it is known from other tumor syndromes (for both benign and malignant tumors) that the mechanism of the second "hit" can be methylation-based silencing instead of a primary mutation. My graduate student Lauren Fishbein is finishing a project employing the sodium bisulfite sequencing method to assay for CpG methylation in a set of LOH-negative plexiform neurofibromas at the promoter region. This surveys a 400 bp region spanning the transcription start (screening 35 CpG dinucleotides), where the expected "normal" pattern of NF1 gene methylation has already been established in non-Schwann cells and crosses from normally methylated to normally unmethylated. We are aiming to complete sequencing of at least 10 clones from each of 15 tumor samples (9 solid, 4 culture) plus normal (non-NF1) Schwann cells, and only need a few more clones from 2 samples. Ten of the samples showed some methylation, although at lower levels than one normally sees in

cancer. The percentage of clones showing at least one methylated site ranged from 10-50%; a maximum of 50% would be expected since methylation would not be expected on the germline mutant allele. Although most of these clones only showed a few methylated sites, it was intriguing that in 7 tumors the same putative AP2 site was affected. This may suggest at least a partial transcriptional effect, which could be followed up in later work using these data as preliminary work for future grants. Ms. Fishbein presented her data in a poster at the 2002 NNFF International Consortium meeting in June (Aspen, CO)(abstract listing in bibliography). Methylation is of special interest to cancer geneticists because the possibility exists of reversing methylation-based silencing, re-activating a normal allele.

Progress on Task A4: NF1 tumor samples and derivative cultures will be analyzed using immunocytochemical and Western blot analyses to determine if they are indeed devoid of neurofibromin.

Completed, except for the three new cultures that have only recently been established. Previous samples were summarized in this year's publication (Muir et al., 2001, Appendix) of finding of neurofibromin-deficiency in 18 Schwann cell cultures derived from neurofibromas (both dermal and plexiform, by Western blot). The other cultures (which did not show such strong enrichment for Schwann cells, or seem to have a cell type that is not typical Schwann) do show some neurofibromin upon Western blot, which is harder to interpret due to the culture characteristics. Western blot analysis of three other new cultures indicates neurofibromin deficiency. We have also immunostained over 40 dermal neurofibroma and 30 plexiform NF1 neurofibroma sections with the Santa Cruz N-terminal polyclonal NF1 antibody (corresponding to a peptide encoded within exon 10a), and have found that all have at least patches of neurofibromin-deficient Schwann cells (some completely devoid of neurofibromin staining within the tumor section), and some of these data are included in the above paper. This is consistent with the genetic data supporting the two hit hypothesis in all NF1 neurofibromas. Of note is that neurofibromin Western blots always shows additional bands below the neurofibromin area, as has been observed by other labs using this and other antibodies. Thus, Dr. Muir and I created of a new antibody to see if we can find one that is clearer on Western blots and useful for other applications. A peptide corresponding to a hydrophilic stretch of 14 amino acids encoded within exon 2 (thus, very N-terminal, residues 27-41) was used to for monoclonal and polyclonal antibody production. Of the two monoclonals derived, one works reasonalby well on Western (but still shows lower bands, NFn27a) and the other is not very good for Western but is very strong on immunohistochemistry (NFn27b). The polyclonal antibody is adequate for Western (no better than Santa Cruz) but also stains fixed sections well. With the DOD's permission, these antibodies are now being produced and licensed so that other researchers can access them (we also sent some free aliquots to collaborators in the earlier stages of development).

Progress on Task A5: Complete immunocytochemistry analysis for neurofibromin protein of tumors/cell cultures; complete Western blot studies for neurofibromin on tumors/cell cultures.

Completed--see Task A4.

Progress on Task A6: Complete antisense inactivation of NF1 to model NF1 inactivation in NF1-relevant cells; perform tumorigenicity related assays in antisense inhibited cells.

Completed. As discussed previously, oligonucleotide antisense inhibition was abandoned in favor of stable transfection with plasmid constructs. In the previous year, an antisense construct of 450 bp of human NF1 cDNA (corresponding to exons 4a-6) in an inducible Invitrogen expression vector failed to express and perform as hoped in rat

Schwann cells as a test of the system. Thus, two new inserts, both of which contain the natural NF1 translation start sequences (one extends into exon 3, the other into exon 7), were amplified last year from rat RNA, and cloned into the same inducible transfection system. Transfection and selection was completed for controls and experimentals, and cells (including some clones isolated from the experimental plates) were harvested for protein and RNA analysis. The RT-PCR assays were reliable and reproducible, including housekeeping and other controls. However, the data showed that none of the antisense clones had more than a 15% reduction in NF1 mRNA, using semi-quantitative image analysis and multiple replications and gel loadings. The Western blot analysis however showed a greater loss in neurofibromin, and we cannot immediately explain this discrepancy. However, the clones do not appear to be null and so are not useful for the planned tumorigenicity assays or other future work since they are no more deficient in neurofibromin than NF1 patient heterozygous Schwann cells. Since antisense approaches have not generally been reported for genes as large as NF1, it is possible that there are underlying mechanisms that effectively prohibit successful antisense inhibition for this gene and tolerate some degree of double stranded RNA (perhaps supported by the fact that some tissues coordinately express both NF1 and embedded genes EVI2A, EVI2B, and OMGP simultaneously; and RNA-structure analytical programs show strong secondary nature in the NF1 transcript, with lots of stem/loops). Thus, despite multiple attempts including completion of the last experiments, we were unable to generate an adequate antisense effect.

<u>Technical Objective B</u>. Evaluate the Involvement of the TP53 Gene in NF1 Tumors.

<u>Progress on Task B1</u>: Complete TP53 LOH studies on all available tumors.

Completed; see manuscript in Appendix (Rasmussen et al., 2000). TP53 LOH was only found in MPNSTs, not benign NF1 tumors.

<u>Progress on Task B2</u>: Complete TP53 sequencing on all tumors.

Completed; see manuscript in Appendix (Rasmussen et al., 2000). No specific TP53 mutations were found in exons 4-9, the most commonly mutated exons, including in MPNSTs.

<u>Progress on Task B3</u>: Complete inactivation of TP53 by antisense technology in Schwann cells; perform transformation related assays in TP53 antisense inhibited cells.

As described previously, this Task was not pursued further since TP53 is only involved in MPNSTs. Also, the NF1/TP53 cis double knockout mice (Drs. Tyler Jacks and Luis Parada) provide an MPNST system that supplants an antisense approach.

<u>Progress on Task B4</u>: Perform immunocytochemistry studies for p53 on all tumors.

Completed. No tumor sections (MPNSTs plus several plexiforms) showed accumulated p53 protein. This is consistent with the lack of point mutations in these samples. However, in the literature there are a few MPNSTs reported with TP53 point mutations, and so it is clear that the involvement of TP53 is somewhat heterogeneous in MPNSTs. This is also consistent with our observation of abnormal karyotypes in just a proportion of plexiforms and MPNSTs (and even then, the abnormalities are not common between samples). Clearly the human tumors are heterogeneous not only for NF1 mutations (with a common feature seeming to be inactivation of neurofibromin through various means), but also for other genetic events, including those outlined below in Objective C.

Technical Objective C. Examine the Involvement of Genes other than NF1 and TP53 in NF1 Tumorigenesis.

Progress on Task C1: Complete cytogenetic analyses on neurofibroma and neurofibrosarcoma cell cultures.

Completed. We had previously reported that two-thirds of plexiform Schwann cultures had abnormal karyotypes; none were found in 7 dermal cultures (Wallace et al, 2000; see Appendix). The abnormalities were not consistent between the samples, and 3 samples had multiple different clones suggesting ongoing evolution of the tumor and possibly chromosomal instability in some. We obtained karyotypes on four of the new Schwann cell cultures derived during this grant, and found that 3/3 plexiforms and 1/2 MPNST lines had a normal karyotype. This latter observation is interesting in light of the fact that the MPNST culture with normal chromosomes has a higher proliferation rate in culture, and the opposite is true of the other culture which shows multiple different clonal abnormalities (e.g. lack of a sex chromosome in all cells, in some cases the X and sometimes the Y). These cytogenetic data will be incorporated in papers during this next year reporting collaborative analyses between Dr. Muir's lab (cell biology/xenoplant system) and my lab (genetics data). One of our newest cultures (tumor received this summer) is an MPNST which we hope our cytogenetics collaborators will karyotype to add to these data.

Progress on Task C2 : Perform comparative genomic hybridization (CGH) on 5 cutaneous, 5 plexiform tumors and 5 neurofibrosarcomas.

Completed. Dr. Deborah Marsh (Kolling Inst. of Medical Research, Sydney, Australia) analyzed DNA, using this method, from 18 tumor DNA samples (11 plexiform; 5 dermal; 2 MPNST)(most of which are culture DNAs, not solid tissue DNAs, some LOH + and some LOH--). We chose to focus more on plexiform tumors and less on MPNSTs, compared to the original plan, based on our finding of cytogenetic abnormalities in some plexiforms and the general lack of information in the literature about plexiforms. Of the 18 samples, 10 showed no detectable gains (cytogenetic level). Of the other 8, 6 had a gain of chromosome 19 material (1 dermal, 3 plexis, 2 MPNSTs). One MPNST had a gain on chromosome 7, consistent with the fact that cytogenetics showed that that tumor culture had trisomy 7 among a few other abnormalities. None of the samples showed DNA losses using CGH, although one LOH-positive dermal culture may have a loss on 16 and 19 (signals not clean enough to be sure), and another LOH-positive dermal culture also looks suspicious for a loss on chromosome 16. The data cannot specify segments of chromosomes, and cannot even specify which arm for the smaller chromosomes. In general with CGH, it is easier to detect gains than losses, but that may relate to the gains often being multiple copies of genes such as oncogenes, whereas losses are merely a 50% drop in material. The repeated detection of chromosome 19 suggests that a sequence/gene/region may be amplified in some NF1 tumors. We are examining the databases to survey for known tumor-promoting genes that lie on 19. Several oncogenes are reside on chromosome 7 (as well as the multi-drug resistance gene) and it is possible that these may be implicated in that MPNST. We are considering submitting these data for publication, and using this work as preliminary data for further funding to follow up these clues.

Progress on Task C3: Perform LOH studies for other tumor suppressor loci on NF1 tumors.

Completed. A tetranucleotide microsatellite marker in NF2 (intron 1) detects LOH in 5/8 MPNSTs and 3/43 informative plexiform tumors. No dermal tumors (n=23 informative situations) have shown NF2 LOH. We are finishing genotyping of an NF2 RFLP in the 3' half

of the gene (an AccI SNP in intron 12), to test for the extent of NF2 LOH and screen more tumors by virtue of this marker being informative in some samples homozygous for the tetranucleotide. These data are intriguing and build upon recent suggestions in the literarue that the NF1 and NF2 proteins have functional significance in some of the same pathways. This makes some sense in that Schwann cell tumors are a cardinal feature of both conditions, and suggests that NF2 loss may be a factor in plexiform neurofibroma and MPNST progression. As a side note, one NF2 schwannoma and 3 schwannomas from a patient not meeting NF2 diagnostic criteria also showed LOH for the microsatellite. A paper presenting these LOH observations is in preparation. The p16 (CDKN2A) and TP73 LOH analyses were performed on plexiforms and MPNSTs, with no evidence of LOH except one MPNST that showed TP73 LOH. However, this marker is not very informative and thus only 15 samples were able to be analyzed. It is interesting that the p16 gene was negative given the report of this gene's LOH in some MPNSTs, but it is likely just a further verification of the genetic heterogeneity that we are observing in NF1 tumors. An RB1 marker failed to detect LOH in a 25 plexiform and 15 informative dermal neurofibromas, although one MPNST was positive for LOH (not the same tumor as the TP73 loss above). Although not originally planned, my undergraduate research volunteer will also do an LOH analysis of the PTEN gene, which has recently been implicated in Proteus syndrome and a growing list of tumor types including lipomas. She will complete this in the next few months. Our data are consistent with LOH of other genes (especially on chromosomes other than 17) tending to occur only in plexiforms (rarely) and MPNSTs (more commonly), which are much more complex tumors than dermal neurofibromas. This is consistent with a theory that dermal neurofibromas may have a clonal Schwann cell element that carries only NF1/chromosome 17 mutations, which by themselves or in conjunction with extrinsic factors (e.g. growth factors, hormones) initiate dermal neurofibroma formation. In contrast, plexiform tumors and MPNSTs (both much larger, and MPNSTs typically arise from plexiforms) show a greater frequency of additional primary genetic alterations, which are heterogeneous but may all ultimately have the same result of accumulated cell mass.

<u>Progress on Task C4</u>: Perform second CGH series on tumors of interest (probably all MPNSTs, unless alterations identified in plexiforms in first series of CGH).

See Task C2.

<u>Progress on Task C5:</u> Submit samples for differential display (DD) analysis to Core Lab.

Completed, with 22 bands sequenced by the UF Differential Display Core. None of the genes identified by differential display also came up as the best candidates by cDNA expression arrays (see paragraph below), or were on the array membranes/chips. This past year we created a custom spotted array of the clones using a robotics station, and hybridized duplicate membranes to P-33-labelled cDNA from the same tumor RNA samples as used for DD and expression analysis. The data were not entirely convincing due to the weakness of the signals (thus, poor sensitivity in detecting small RNA level changes). Only two sequences appeared to have reproducible data (ESTs), with one moderately increased in quantity, and the other decreased in tumors relative to normal Schwann cells, although not dramatically. Neither EST has a genome match with a known gene, but represent an open reading frame of over 100 bp, and thus these may be novel genes. Further analysis would be necessary to confirm that these are genes and are truly consistently altered in neurofibromas. This seems unlikely to pan out given that the other DD clones seem to have been false positives.

Because of the false positive problem known in the DD system, we developed an alternative approach (array expression analysis) in parallel, which is now completed. Nine

plexiform tumor culture RNAs were hybridized to the Clontech Atlas Cancer cDNA array membranes, compared to normal Schwann cell RNA, to identify aberrantly expressed transcripts (the array has over 1100 genes known to be involved in cancer-related systems such cell cycle, proliferation, apoptosis, DNA repair, etc.). Another set of hybridizations was performed with 3 other samples but the controls were not consistent and so those hybridizations were not considered reliable. In addition, the U.F. Shands Cancer Center awarded us 5 Affymetrix Human Cancer Chips (plus quality control chips), and we analyzed 3 of the same tumor RNAs plus a new one (and the same normal Schwann cells as used for Atlas analysis). The Affymetrix chip contained match and mismatch oligos for over 2000 cancer-related genes (many identical to those on the Atlas membrane), and is a more sensitive system because of its fluorescence-based detection. As expected, the tumor samples are not dramatically different than normal Schwann cells, and there is substantial heterogeneity between samples (not unexpected, given that these are from different human beings and stochastic somatic events are involved). As seen in the attached manuscript (which is nearly ready for submission; Thomson et al.), 9 genes survived a stringent filter and validation through real-time PCR: TIMP3, TSP2, NGFR, TRAIL, HYAL1, HCG, INTB8, GFRA2, MT1. These genes were altered in at least 3/9 tumors (fairly consistently based on patient gender, interestingly), and fall into functional categories related to apoptosis or extracellular matrix metabolism, consistent with cell accumulation. Ms. Thomson presented these data in a talk at the 2002 NNFF International Consortium meeting in Aspen (see bibliography for abstract reference). An attempt to run a dendritic program on the Affymetrix results failed due to lack of samples/heterogeneity. Overall the data suggest that multiple pathways can be involved in tumorigenesis, and these data will provide preliminary data for future grants to further investigate the roles of these molecules and pathways in NF1 tumors. It is interesting to speculate that future therapies might require some characterization of individual tumors to help choose the best drugs/treatment strategies based on their intrinsic abnormalities, should any universal pathways (are there any besides increased ras activity?) fail to prove useful for therapies. These data were included as preliminary work for a collaborative DOD grant application submitted this fall by Dr. Ratner, on which we are the primary site that will provide plexiform cell culture materials for a large-scale gene expression study to identify altered biochemical pathways in NF1 tumors via human and mouse tumor studies.

Although not assayed through the array system, we were also interested in whether neurofibromas expressed telomerase, known to be associated with the immortalization phenotype in cancers. An RT-PCR experiment using silver staining hinted that only MPNSTs express telomerase, not plexiform tumors. However these primers would not work on the real-time PCR system at hand (Roche LightCycler) despite multipe attempts. A collaborating lab here at UF may run telomerase assays on MPNST and plexiform cells. Since telomerase is a target in other cancers, characterization of its presence/absence in NF1 tumors would help determine if those therapies are appropriate in NF1.

As a related project, a mutation analysis was also done to search for a specific activating mutation in the ERBB2 oncogene (transmembrane domain) that is reported in connection with development of Schwann-based tumors in young rodents treated in utero with mutagenizing agents (reviewed by Nakamura, 1995) and also in spontaneous Schwann cell tumors in domesticated animals (Stoica et al., 2001). ERBB2 signals through the ras pathway, and this receptor may also help bind to ligands and transmit signals relevant to Schwann cell proliferation (e.g. glial growth factor 2). Thus, we screened for this mutation in NF1 and non-NF1 neurofibromas (and schwannomas), but failed to find any mutations (there are no reports of activating human ERBB2 mutations, just amplification of the gene in cancers such as breast). However, the PCR assay spanned a known coding region missense

polymorphism that is associated with breast cancer, and thus we genotyped this RFLP in a set of NF1 patients vs. normal controls and were surprised to find a statistically-significant under-representation of the rare homozygous genotype in NF1 patients (277) compared to controls (290). These data were presented at the American Society of Human Genetics meeting in 2001 (Fishbein et al., 2001), and a manuscript was just submitted (Fishbein et al., Appendix). A polymorphism at another locus was genotyped to ensure that there were no underlying ethnic differences accounting for this observation. The interpretation is not entirely clear but the relationship is intriguing—it could suggest that there is an increased mortality in NF1 embryos homozygous for the rare allele. Alternatively, that genotype might attenuate NF1 and make it less likely to have features which bring a patient to medical attention/diagnosis. Either interpretation suggests that ERBB2 is a possible NF1 modifier gene. Ms. Fishbein's success in this and the methylation work helped her win an NIH NRSA fellowship which recently began, to fund the rest of her PhD thesis work (studying steroid hormone receptor genes in NF1) and her MD training.

<u>Progress on Task C6</u>: Characterize and analyze specific genes suggested by CGH and DD experiments.

See Task C4 and C5. The data from the CGH and expression array studies will be preliminary data for future grants to investigate the role of the implicated proteins and test for which loci are amplified in the tumors. The two DD-implicated ESTs may also be pursued in future grants, although the likelihood that they are important in NF1 is low.

KEY RESEARCH ACCOMPLISHMENTS

<u>For Technical Objective A (establish two-hit hypothesis in NF1 gene in NF1 tumors):</u>

- Task A1: Completed.
- Task A2: Completed.
- Task A3: Completed.
- Tasks A4 and A5: Completed.
- Task A6: Completed attempts.

<u>For Technical Objective B (study involvement of TP53 gene in NF1 tumors):</u>

- Task B1: Completed.
- Task B2: Completed.
- Task B3: Dropped.
- Task B4: Completed.

<u>For Technical Objective C (examine involvement of genes other than NF1 and TP53):</u>

- Task C1: Completed.
- Task C2 and C4: Completed.
- Task C3: Completed.
- Task C5 : Completed.
- Task C6: Completed.

REPORTABLE OUTCOMES

1. The tumor Schwann cell culture technique, the finding of neurofibromin-negativity in cultures by Western, and the ability of these cultures to survive and grow as sciatic nerve xenoplants in scid (immunodeficient) mice was published (Muir et al., 2001). This latter finding was the basis for 2 currently-funded DOD grants (Dr. Muir as PI of one, myself as PI of another to study steroid hormones in NF1).

2. A manuscript reporting the aberrant NF1 splicing data was published this year (Thomson et al., Human Genetics 2002)

3. Discovery of numerous germline NF1 mutations, and 7 specific somatic mutations in tumors, as well as NF1 LOH analysis, was in part reported in 2000 (Rasmussen et al), 2002 (Thomson et al., Journal of Child Neurology), and a planned manuscript.

4. The isodisomy finding will be confirmed with a final experiment to produce data sufficient for a publication.

5. The monoclonal and polyclonal amino-terminus neurofibromin antibodies are licensed and available.

6. Methylation analysis of NF1 tumors has yielded positive data which will be submitted for publication within the next few months, having been presented in poster form at the 2002 NNFF International Consortium meeting in Aspen in June. This work may provide preliminary data for future grants.

7. Array expression data on plexiform Schwann cell cultures will be submitted as a manuscript within the next month. Some of these data were presented as a poster at the American Society of Human Genetics meeting in October 2001, and final data were then presented as a talk at the International NF Consortium meeting in 2002, by graduate student Susanne Thomson. This work will be important in future grant applications to study regulation of apoptosis and extracellular matrix in NF1 tumors.

8. ERBB2 mutation/genotyping data were presented as a poster by graduate student Lauren Fishbein at the American Society of Human Genetics meeting in 2001, and were submitted as a manuscript recently, with the inclusion of a another gene analysis to rule out ethnic background differences.

9. Some of the isodisomy and somatic mutation data, including NF2 LOH, were reported by Dr. Wallace at an invited talk at the NNFF International Consortium Meeting on the Molecular Biology of NF1 and NF2, in June 2001, at Aspen, CO. Dr. Wallace co-chaired the meeting this past June and presented some array work, which resulted in a collaboration on a multi-center tumor gene expression grant submitted by PI Nancy Ratner this past September.

10. CGH data are complete and will be compiled into a small manuscript for submission in the next year, integrating other known genetic data such as karyotype information.

LIST OF PERSONNEL RECEIVING PAY FOR RESEARCH EFFORT

Margaret Wallace, Ph.D.
David Muir, Ph.D.
Anthony Yachnis, M.D.
Amyn Rojiani, M.D.
Susanne Thomson (grad. student)
Lauren Fishbein (grad. student)
Marisa Scott (technician)
Eileen Monck (technician)
Rachael Trimpert (technician)
Kyle Roux (technician)
Lisa Jacobs (technician)
Lori Beth Fisher (technician)
Julie Bokor (technician)
Karen Dyshuk, Bonnie Eady (lab support)

CONCLUSIONS

The data substantially support the two-hit hypothesis in NF1 tumors, specifically in a genetically-abnormal clonal Schwann cell population in neurofibromas, with somatic mutations ranging in types. This suggests that loss of neurofibromin is associated with tumorigenesis, although whether this is sufficient for human tumorigenesis is still not completely answered (but it is intriguing that dermal neurofibromas rarely show any abnormalities outside the NF1 somatic mutation, but multiple gene and expression abnormalities are present in many plexiforms and MPNSTs). The NF1 molecular genetic work indicates that caution must be exercised when analyzing NF1 mutations involving splicing, and tumor pathogenesis. Characterization of the cell cultures and the primary tissues using the approaches employed here have gained much information about the types of abnormalities and the frequencies/consistencies which will help in therapeutic design, aimed perhaps at replacement of neurofibromin or one of the molecules/paths implicated through research such as ours. Our mutation data may shed light on possible functional domains of neurofibromin, which might allow for compensation for one or more functions without the need to replace the whole molecule. Since plexiform neurofibromas are much more medically significant than dermal and affect more patients than MPNSTs, our focus has been on this tumor type in most of the experiments. This complements the Army's NF program initiatives in natural history studies of plexiforms, and the few clinical trials, which mostly focus on plexiforms. We now have excellent clues about biochemical abnormalities in the different tumor types, and the heterogeneity present in the population. This will launch future directions in biochemistry and pharmaceutics aimed at treating NF1.

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Invited talk by Dr. Wallace: "Gene Expression Analysis in Human Plexiform Neurofibroma Schwann Cell Cultures." Microarray satellite meeting at the NNFF International Consortium on the Molecular Biology of NF1 and NF2, Aspen, CO, June 2002.

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APPENDIX

Reprints/preprints of articles:

- Fishbein et al., submitted.
- Muir et al., 2001
- Rasmussen et al., 2000
- Wallace et al., 2000
- Messiaen et al., 1999
- Thomson et al., 2002a
- Thomson et al., 2002b
- Thomson et al., draft

An Association Between the *ERBB2* Codon 655 Polymorphism and NF1

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ERBB2: Modifier of NF1?

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ABSTRACT

Neurofibromatosis 1 (NF1) is an autosomal dominant disorder characterized by cellular overgrowth of neural crest derived tissues, a key feature being benign Schwann cell tumors called neurofibromas. NF1 is caused by mutations in the *NF1* gene. There is variable expressivity, and it is thought that other genes likely modify the NF1 phenotype. The proto-oncogene *ERBB2* (a.k.a. *HER2/neu*) encodes a tyrosine kinase receptor, which is a member of the epidermal growth factor receptor family. ErbB proteins have been implicated in animal neurofibroma development, via a specific activating *ERBB2* mutation. However, no human tumors have been reported with this mutation, including 29 NF1 tumors and 19 non-NF1 Schwann cell tumors examined in this study. An Ile/Val polymorphism exists at codon 655 in the transmembrane domain near the mutation and is associated with an increased risk for breast cancer. The role that the *ERBB2* gene product plays in cell proliferation, as well as the critical function of the transmembrane domain in receptor activation, make this polymorphism a candidate for a modifier gene for NF1. We examined the genetic association between the *ERBB2* codon 655 alleles and NF1. Among 277 NF1 patients and 290 controls, we found that the risk of having NF1 with the Val/Val genotype was lower than with the Ile/Ile genotype. Furthermore, the number of NF1 patients with a homozygous Val/Val genotype is significantly lower than that found in our control populations. These data, the first report of a genetic association analysis of NF1, suggest that there may be a biological relationship between *ERBB2* genotype and NF1 phenotype.

Key words: Neurofibromatosis 1 (NF1), *ERBB2*, polymorphism, association, modifier gene

INTRODUCTION

Neurofibromatosis 1 (NF1) affects approximately 1 in 3500 individuals, and is characterized by benign overgrowth of neural crest derived tissues, including the formation of café-au-lait spots, Lisch nodules and neurofibromas. Neurofibromas are slow-growing tumors of the peripheral nerve sheath, composed mainly of Schwann cells. Some patients may have only a few small dermal neurofibromas, while other can develop thousands and/or have large neurofibromas from on major nerve branches. Clinical symptoms are highly variable between patients, even within families, but this is not obviously correlated to NF1 mutation type. Consequently, it is thought that along with stochastic factors, other genes may play a role as modifiers in NF1 phenotype (Easton et al., 1993).

The proto-oncogene *ERBB2* (or *HER2/neu*) encodes a transmembrane tyrosine kinase receptor and is a member of the epidermal growth factor receptor family thought to control a number of cellular functions, such as cell proliferation (reviewed in Dougall et al., 1994). The receptor heterodimerizes with other members of the ErbB family, activating the tyrosine kinase domain and transmitting growth factor signals downstream to cytoplasmic targets, including the MAPK pathway (which is also downstream in the *NF1* pathway). These growth factors include ligands known to stimulate Schwann cell proliferation. The *ERBB2* gene is frequently amplified or over-expressed in breast cancer and a number of adenocarcinomas, often signifying a poor prognosis (reviewed in Dougall et al., 1994). Transplacental administration of *N*-nitroso-*N*-ethylurea (ENU) to rats and hamsters induces multiple tumors in offspring at a high incidence (Bargmann and Weinberg, 1988; Nakamura et al., 1994). The rat Schwann cell tumors and hamster neurofibromas contain an activating point mutation in the *ERBB2* transmembrane domain (e.g. rat codon 664; analogous to codon 659 in humans and hamsters), leading to a valine

to glutamic acid amino acid substitution. This mutation is believed to cause an increase in the tyrosine kinase activity, and the mutated gene has been shown to transform NIH 3T3 cells (Bargmann et al., 1986).

To our knowledge, no human tumors have been found with this mutational change, although a coding region polymorphism has been identified in the transmembrane region (Papewalis et al., 1991). The polymorphism is located at codon 655 in humans and encodes either an isoleucine (ATC) or valine (GTC). It is unknown whether the polymorphic proteins differ in their function or tyrosine kinase activity. Interestingly, results from a population-based, case-control study examining the association of the *ERBB2* polymorphism with the risk of breast cancer showed a statistically significant increased risk for early onset breast cancer in women homozygous for the valine allele (Xie et al., 2000). To search for a connection between *ERBB2* and NF1/Schwann cell tumors, we first screened for the codon 659 mutation in a set of tumors, and then searched for a genetic association between the *ERBB2* codon 655 alleles and NF1.

MATERIALS AND METHODS

Patient Populations.

Case samples consisted of unrelated NF1 patient leukocyte DNA previously collected under IRB approval in our laboratory (n= 180) and an anonymous set generously provided by Dr. Karen Stephens (n=97) (University of Washington, Dept. of Pathology). The NF1 diagnosis was made via clinical observations satisfying the NIH diagnostic criteria. These patients were ascertained through genetics or other clinics, or via advertising through the National Neurofibromatosis Foundation. Two control groups were obtained, with the first group (C1) consisting of unrelated non-NF1 individuals whose leukocyte DNA was collected previously in

our laboratory (n=152). The second group (C2) consisted of a panel of anonymous control (non-NF1) DNAs generously given to us by Dr. Lawrence Brody (n=138) (National Human Genome Research Institute, NIH) (Struewing et al., 1995). DNA extractions were performed using the PUREGENE DNA Isolation Kit (Gentra Systems). The NF1 and control samples in our laboratory consist mostly of persons of a Caucasian background from the state of Florida.

The *ERBB2* codon 659 mutation analysis studied DNA extracted from primary tumor samples previously collected in our laboratory. The sporadic neurofibromas are from patients who do not meet the diagnostic criteria for NF1, while the NF1 neurofibromas and MPNSTs (malignant peripheral nerve sheath tumors) are from patients who do meet the diagnostic criteria for NF1. The peripheral, cutaneous schwannomas are from both NF2 and non-NF2 patients.

Mutation Analysis.

A 144bp fragment of the *ERBB2* gene was PCR amplified from tumor DNA using primers from Nakamura et al (1994) (NeuA 5'-AGAGCCAGCCCTCTGACGTC, NeuB 5'-CGTTTCCTGCAGCAGTCTCC) and standard conditions with a 58°C annealing temperature. These products were sequenced using standard cycle-sequencing protocols in our lab (ABI BigDye Terminator Kit) and results analyzed with the SeqEd v1.0.3 program (ABI).

Genetic Polymorphism Analysis.

Genotypes for *ERBB2* were determined with a PCR-RFLP based assay. The primers were designed to amplify the transmembrane domain and were based on the published sequence for the *ERBB2* gene (ErbB2-1F 5'-AGAGTAGGAGAGGGTCCAAGCC-3') or taken from Nakamura et al (1994) as for the mutation analysis (NeuA, NeuB). The PCR amplification was

performed under standard conditions with an annealing temperature of 69°C for the ErbB2-1F/NeuB primer pair and 58°C for the NeuA/NeuB primer pair. The PCR products were then digested with the restriction enzyme BsmBI (NEB). The completely uncut product represents a homozygous isoleucine genotype, while the completely cut product represents the homozygous valine genotype. The digest products were separated with 8% native PAGE and stained with ethidium bromide for visualization. Direct DNA sequencing was performed on some samples to confirm the results for the RFLP analysis.

Due to the lack of detailed information on patient ethnicity, a well-characterized insertion/deletion polymorphism in intron 16 of the human angiotensin converting enzyme (*hACE*) gene was used to provide evidence that ethnic background differences of the NF1 and control populations were not contributing to *ERBB2* differences. Genotypes were determined with a PCR assay using primers from Lindpaintner et al (1995) (hACE3-F 5'-GCCCTGCAGGTGTCTGCAGCATGT-3' and hACE5-R 5'-GGATGGCTCTCCCCGCCTTGTCTC-3'). This product was amplified under standard PCR conditions with an annealing temperature of 58°C. This primer set amplifies both the insertion and deletion alleles, 597 bp and 319 bp respectively. Because the deletion allele can be preferentially amplified in a heterozygous sample, an insertion-specific primer set was used to confirm the absence or presence of this larger allele in samples which were found to be homozygous for the deletion allele (hACE5-F 5'-TGGGACCACAGCGCCCGCCACTAC-3' and hACE5-R 5'-TCGCCAGCCCTCCCATGCCCATAA-3') (Lindpaintner et al., 1995). This product was amplified with a 1 min denaturing step at 94°C and a combined annealing and extension step for 1 min at 72°C, for 35 cycles. PCR products were electrophoretically separated on 1% agarose gels and visualized with ethidium bromide staining.

Statistical Analysis.

Statistical analysis (odds ratios and 95% confidence intervals) was used to determine a possible correlation between *ERBB2* genotype and NF1. Odds ratios (reported as cases/controls) are written as the ratio(95% confidence interval). Hardy-Weinberg analysis and chi-squared analysis for differences in genotype frequencies were completed as well. All statistics are reported based on the comparison of the NF1 group to each control group separately, as well as combined.

RESULTS

Ten sporadic (non-NF1) neurofibromas, 9 Schwannomas, 20 NF1 neurofibromas and 9 NF1 MPNSTs were analyzed for the presence of the *ERBB2* codon 659 missense mutation in the transmembrane domain, and no mutations were found.

Figure 1 shows genotyping from the ErbB2-1F/Neu B PCR product. All three genotypes are represented and the results matched those from the direct sequencing data. A homozygous Ile genotype shows an uncut band at 280 bp (patients 1 and 5). The heterozygous Ile/Val genotype shows one 280 bp band and two lower bands of 165 bp and 115 bp (patients 2, 3, 4). The homozygous Val genotype shows the two lower bands only (patient 6).

Genetic analysis was completed on 277 unrelated NF1 patient samples and 152 C1, 138 C2, and 290 C1 and C2 combined samples (Table 1). The Val allele was less prevalent among NF1 patients (18%) than among either C1 (26%), C2 (26%) or the combined controls (26%). The odds ratios for the allele count are given in Table 1. The risk of having NF1 with the

Val/Val genotype is lower [OR=0.27(0.12-0.65)] than that with the Ile/Ile genotype [OR=0.63(0.44-0.88)].

In addition, the number of samples with a Val/Val genotype is significantly lower in the NF1 population than in the control populations (C1, p=0.001; C2, p=0.002; C1+C2, p=0.002). In contrast, the frequencies for the remaining genotypes are not significantly different among the two populations (Ile/Ile: C1, p=0.120; C2, p=0.086; C1+C2, p=0.111 and Ile/Val: C1, p=0.480; C2, p=0.390; C1+C2, p=0.451). Hardy-Weinberg equilibrium analysis showed that there were small differences between the observed and expected numbers of people in each genotype, but the differences were not statistically significant using chi-squared tests for independence (NF1, p=0.983; C1, p=0.587; C2, p=0.822; C1+C2, p=0.590).

The human angiotensin converting enzyme (*hACE*) gene has a well-characterized insertion/deletion polymorphism in intron 16 for which the genotype frequencies vary based on ethnic background (Mathew et al., 2001). Genotyping of the *hACE* gene in both NF1 and control populations showed no statistically significant difference in genotype frequencies between the populations (Ins/Ins: C1, p=0.870; C2, p=0.729; C1+2, p=0.869 and Ins/Del: C1, p=0.751; C2, p=0.411; C1+2, p=0.671 and Del/Del: C1, p=0.620; C2, p=0.232; C1+2, p=0.533). Allele frequencies also showed no statistically significant difference (data not shown). This implies that the statistically significant differences in genotype frequency seen for the *ERBB2* gene are not a result of ethnic differences between the groups.

DISCUSSION

The *ERBB2* gene product, p185, is a 1255 amino acid glycoprotein that is closely related to the other members of the EGFR family involved in signal transduction. These receptors share

a similar structure consisting of an extracellular ligand-binding domain, a single hydrophobic transmembrane domain, and a cytoplasmic tyrosine kinase domain. Alterations in the transmembrane segment may have profound effects on the receptor activity. The ENU-induced valine to a glutamic acid mutation at rat codon 664 (analogous to human and hamster codon 659) in the transmembrane domain is believed to enhance receptor dimerization, which results in the activation of the receptor tyrosine kinase without ligand binding. NMR studies have shown that this activating mutation can cause significant intramolecular rearrangements which could influence its lateral associations (Sharpe et al., 2000). In addition, *in vitro* studies have shown that activation of *ERBB2* is sufficient to initiate the immortalization and transformation of immature Schwann cells, as well as NIH 3T3 cells (Sherman et al., 1999; Bargmann et al., 1986).

A recent report analyzed a series of spontaneous peripheral nerve sheath tumors from domesticated animals and found the same *ERBB2* mutation in 75% of the malignant tumors (Stoica et al., 2001). Although a homologous mutation has not been found in any human tumors, including our sample set of 48 peripheral nerve sheath tumors, there exists an Ile/Val polymorphism nearby at codon 655 in the transmembrane domain. It is not known if this substitution results in any change in the tyrosine kinase activity. Nevertheless, our study has found that the number of NF1 patients with a homozygous Val genotype is significantly lower than that found in our control populations. This finding suggests that the polymorphism may be an NF1 modifier gene (or linked to a modifier). Interestingly, the heterozygous Ile/Val genotype is not significantly lower in the NF1 population. One possible explanation is a selective embryonic mortality associated with the combination of an NF1 mutation and an *ERBB2* homozygous Val genotype. Heterozygous NF1 cells have decreased NF1-GAP activity and thus have increased signaling through the ras pathway (Kim et al., 1995). This signaling cascade

connects to the MAPK pathway through which ERBB2 signals. Perhaps downstream alterations in MAPK signaling by a combination of *NF1* mutations and homozygosity for *ERBB2* codon 655 valine allele may predispose for a critical effect in development. Alternatively, the Val homozygous genotype at ERBB2 can attenuate NF1 phenotype, such that persons with an *NF1* mutation and Val/Val *ERBB2* genotype might have a greater chance of failing to display sufficient features to be diagnosed and/or brought to medical attention. However, of the 4 (out of 7 total) Val/Val NF1 patients for which phenotype information is known, two each have a serious medical complication; and the other two are only mildly affected, although the serious complications might be due to stochastic factors. Thus, these data support the notion that *ERBB2* genotype may have a biologically-based relationship to NF1 phenotype through mechanisms which bear further investigation.

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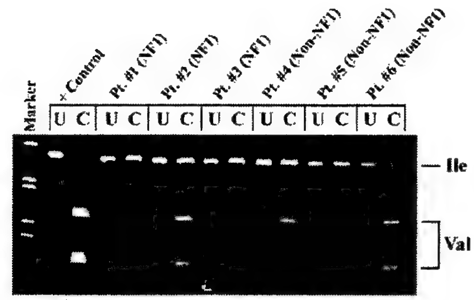
Table 1. ***ERBB2*** **allele frequencies and odds ratios [OR=Odds ratio(95%Confidence Interval)] for the NF1 and control group populations.**

	NF1 patients	C1	C2	C1 and C2
	n=277	n=152	n=138	n=290
Allele Frequency, %				
Ile	82	74	74	74
Val	18	26	26	26
Genotype Frequency, %				
Ile/Ile	68	57	56	57
Ile/Val	30	34	36	35
Val/Val	3	9	8	9
ORs for Allele Count				
cases/controls		0.60(0.43-0.85) p=0.003	0.60(0.43-0.85) p=0.004	0.60(0.45-0.80) p=0.001
Homozygous effect, ORs				
Val/Val		0.26(0.10-0.65) p=0.004	0.30(0.11-.79) p=0.015	0.27(0.12-0.65) p=0.003
Ile/Ile		0.64(0.43-0.97) p=0.035	0.61(0.40-0.92) p=0.020	0.63(0.44-0.88) p=0.007

FIGURE LEGENDS

Fig 1. Ethidium-bromide stained polyacrylamide gel showing genotyping of the *ERBB2* polymorphism. The first lane shows the 1kb ladder marker (Invitrogen). For each sample, the undigested product is shown first (U). The BsmBI digested product is shown second (C), with the top band (280bp) representing the Ile allele and the bottom bands (165bp, 115bp) representing the cut Val allele. Positive control and patient 6 show the Val homozygous genotype.


Marker
+ Control
Pt. #1 (NF1)
Pt. #2 (NF1)
Pt. #3 (NF1)
Pt. #4 (Non-NF1)
Pt. #5 (Non-NF1)
Pt. #6 (Non-NF1)
U C U C U C U C U C U C U C
Ile
Val

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Tumorigenic Properties of Neurofibromin-Deficient Neurofibroma Schwann Cells

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Dermal and plexiform neurofibromas are peripheral nerve sheath tumors that arise frequently in neurofibromatosis type 1. The goal of the present study was to examine the tumorigenic properties of neurofibromin-deficient human Schwann cells (SCs) that were found to represent a subset of SCs present in approximately half of the total neurofibromas examined. Highly enriched SC cultures were established from 10 dermal and eight plexiform neurofibromas by selective subculture using glial growth factor-2 and laminin. These cultures had low tumorigenic potential in classical *in vitro* assays yet several unique preneoplastic properties were frequently observed, including delayed senescence, a lack of density-limited growth, and a strong propensity to spontaneously form proliferative cell aggregates rich in extracellular matrix. Western blot analysis failed to detect full-length neurofibromin in any of the neurofibroma SC cultures, indicating that neurofibromin-deficient SCs had a substantial growth advantage. Immunohistochemical staining of the originating tumors showed the majority were comprised principally of neurofibromin-negative SCs, whereas the remainder contained both neurofibromin-negative and neurofibromin-positive SCs. Lastly, engraftment of neurofibromin-deficient SC cultures into the peripheral nerves of *scid* mice consistently produced persistent neurofibroma-like tumors with diffuse and often extensive intraneural growth. These findings indicate that neurofibromin-deficient SCs are involved in neurofibroma formation and, by selective subculture, provide a resource for the development of an *in vivo* model to further examine the role of these mutant SCs in neurofibroma histogenesis. *(Am J Pathol 2001, 158:501–513)*

Neurofibromatosis type 1 (NF1) is a common autosomal dominant condition with a high frequency of peripheral nerve sheath tumors called neurofibromas. Dermal neurofibromas usually develop during adolescence and adulthood. These small tumors involve terminal nerves and may be numerous, yet have no apparent risk of malignant transformation. In contrast, plexiform neurofibromas are usually congenital, typically involve deep or "named" nerves, can become very large, and may cause serious functional impairment. Because plexiform tumors often occur on critical nerves and are not discrete masses, surgical removal is rarely complete and recurrence is associated with increased morbidity and fatality. A recent study suggests that plexiform neurofibromas develop in the majority of NF1 patients.[1] Additionally, plexiform tumors may progress to malignancy, which occurs in an estimated 6% of NF1 patients.[2]

Unlike schwannomas, which consist predominantly of Schwann cells (SCs), neurofibromas show marked cellular heterogeneity. Nevertheless, SCs are the major cell type amplified in neurofibromas and typically comprise 40 to 80% of the tumor cells. Additionally, there is a substantial population of interspersed fibroblastic or perineurial cells, along with various vascular and inflammatory elements embedded in an extensive extracellular matrix.[3] Because of this cellular heterogeneity, the histogenesis of neurofibromas has been controversial. Although there is increasing evidence for a SC origin, some studies suggest the contribution of SCs and fibroblastic cells.[4–6] On the other hand, an emerging view proposes that all of the major cellular elements of neurofibromas are of SC lineage and that the fibroblastic/perineurial-like elements may be immature or variant SCs.[7–10]

NF1 is caused by disruptive mutations in the *NF1* gene, which encodes the GAP-related protein neurofibromin. Thus, all cells in an NF1 individual are initially haploinsufficient for neurofibromin activity(s). However, consistent with the tumor suppressor gene two-hit model, it seems that loss of function of the remaining *NF1* allele is associated with neurofibroma formation, as first observed by our lab as loss of heterozygosity.[11] Although there are several approaches to animal models of NF1, presently none exist in which neurofibromas can be readily induced using defined human cell populations deficient in neurofibromin. Gene targeting has been used to construct mouse strains harboring mutations in the *Nf1* gene.[12,13] The mouse knockout model, similar to the

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human NF1 condition, involves only a single constitutional mutation and homozygous mice ($Nf1^{-/-}$) die during gestation. Despite the high level of conservation between mouse and human neurofibromin, it is clear that *Nf1* knockout mice are not prone to the formation of neurofibromas. Thus, the inadequacy of the heterozygous mouse model may be attributed to a low mutation rate of the remaining wild-type *Nf1* allele within the relatively short murine life span. Original studies by Martuza and co-workers[14] demonstrated the growth of minced human neurofibromas in the subrenal capsule and sciatic nerve of immunodeficient mice that retained their morphological features and genomic identities. Thus, the use of defined neurofibroma cell populations in animal models will greatly enhance efforts to understand the histogenesis of neurofibromas.

Neurofibroma SCs have invasive and angiogenic properties, suggesting that these are genetically altered cells with tumorigenic properties.[15,16] Additionally, cytogenetic studies show that plexiform neurofibromas harbor genetically abnormal SCs and strongly implicate these cells as the central component in the development of these potentially progressive tumors.[17] Recently, somatic loss of heterozygosity was found in SCs, but not fibroblasts cultured from a neurofibroma, suggesting that genetic alterations of the *NF1* gene in SCs are involved in the development of neurofibromas.[18] In a more comprehensive study, Rutkowski and co-workers[19] further demonstrated that neurofibroma-derived SCs typically lacked *NF1* mRNA whereas fibroblasts isolated from neurofibromas expressed the *NF1* transcript. In the present study, SCs subcultured from numerous neurofibromas were examined for neurofibromin expression and tumorigenic properties *in vitro* and after intraneural engraftment. Our findings strongly implicate neurofibromin-deficient SCs in the histogenesis of at least a subset of neurofibromas.

Materials and Methods

SC Culture

Normal Adult Nerve SCs

All specimens included in this study were obtained in accordance with protocols approved by the University of Florida Institutional Review Board. Human SCs were isolated from normal adult sural nerves by modifications of methods described previously.[20,21] Briefly, segmented nerve fascicles were cultured for 10 days in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 5% calf serum, 2 μm forskolin, 25 ng/ml human recombinant glial growth factor-2 (GGF-2), and antibiotics (expansion medium). The tissue was then dissociated for 18 hours in medium 15% calf serum, 1.25 U/ml Dispase (Collaborative Research Inc., Bedford, MA), 300 U/ml collagenase (type XI; Sigma Chemical Co., St. Louis, MO) and antibiotics. The digested tissue was dispersed by trituration, passed through a 30-μm mesh nylon screen, and centrifuged (200 $\times$ g, 10 minutes). The cell pellet was resuspended (50 segments/2 ml) in medium containing N2 supplements[22] and 2 ml of the cell suspension was spread across the surface of a 75-cm^2 flask precoated sequentially with polyornithine (0.1 mg/ml) and laminin (10 μg/ml) (prepared as described by Muir[23]). After a 6-hour incubation, the medium was supplemented by the gentle addition of expansion medium (10 ml). The cultures were grown to near confluency and the SCs were isolated by differential detachment using mild trypsinization and gentle shaking. The highly enriched cultures were expanded in expansion medium in dishes coated with laminin only. All cultures were withdrawn from treatment with forskolin and GGF-2 before storage or use.

Neurofibroma SCs

All patients met recognized diagnostic criteria for NF1[24] and tumor specimens were characterized as neurofibromas by histopathological study. Patient ages ranged from 4 to 69 years (the majority were young to middle-aged adults) and the reasons for surgery included cosmetic, functional deficit, and tissue donation on NF1-related fatality. These factors were unrelated to the tissue culture outcome and phenotype. Any capsular material was removed and viable tumor isolated from surgically resected neurofibromas. Tumor tissue (1 cm^2) was finely minced and incubated at 37°C overnight in 10 ml of L-15 medium containing 15% calf serum, 1.25 U/ml Dispase, 300 U/ml collagenase, and antibiotics. The tissue was dispersed by trituration and strained through a 30-μm mesh nylon screen. The filtrate was diluted with L-15 and centrifuged (400 $\times$ g, 5 minutes). The cell pellet was resuspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 5% calf serum, and antibiotics (standard medium), and cells were seeded into tissue culture flasks ($\approx 10^6$ cells/75 cm^2. After 4 days, cultures were detached with trypsin/ethylenediaminetetraacetic acid and passaged 1:4. Half of the passaged cells were grown in standard culture conditions; these were later harvested and stored in liquid nitrogen. The other half of the cells were seeded in flasks precoated with laminin (10 μg/ml) and grown in standard medium containing GGF-2 (25 ng/ml). GGF-2 treatment caused rapid proliferation of Schwann-like cells (SLCs). During subsequent passage, SLCs were enriched further by differential detachment using mild trypsinization and shaking. For the specified cultures, the combination of preferential laminin attachment, differential detachment, and selective mitogen treatment with GGF-2 yielded highly enriched (95 to 99.5%) SLC cultures within 3 to 4 passages. All tumorigenic and protein expression assays were performed using cultures at passage 3 to 4 that had been withdrawn from GGF-2 for at least 2 days. GGF-2 was generously provided by M. Marchionni (Cambridge Neuroscience, Cambridge, MA).

Anchorage and Serum Requirements

The growth of SLC-enriched cultures was assessed in serum-free and unattached conditions. Early (passage 2

Table 1. Characteristics of Neurofibroma-Derived Schwann Cell Cultures

SC Culture	Culture type*	S-100/p75†	NFn SC culture‡	NFn tumor§
Derived from dermal neurofibromas				
SC+(cNF89.1)	type-2	+/+	−	n.d.
SC+(cNF93.1a)	type-4	v/v	−	−/+
SC+(cNF93.1b)	type-4	v/v	−	−/+
SC+(cNF96.5f)	type-2	+/+	−	−/+
SC+(cNF96.5g)	type-2	+/+	−	−/−
SC+(cNF97.2a)	type-2	+/+	−	−/−
SC+(cNF97.2b)	type-2	+/+	−	−/−
SC+(cNF98.4a)	type-2	+/+	−	−/−
SC+(cNF98.4d)	type-2	+/+	−	−/−
SC+(cNF99.1)	type-2	+/+	−	−/−
Derived from plexiform neurofibromas				
SC+(pNF92.1)	type-3¶	+/−	−	−/−
SC+(pNF94.5)	type-2	+/+	−	+/−
SC+(pNF95.1)	type-2	+/+	−	−/−
SC+(pNF95.5)	type-3	+/±	−	−/+
SC+(pNF95.6)	type-3¶	+/±	−	+/−
SC+(pNF95.11b)	type-3¶	+/+	−	+/−
SC+(pNF97.9)	type-4	v/v	−	−/−
SC+(pNF98.3)	type-3	+/+	−	−/+

*Growth classification specified in Results.
†Immunocytochemical expression of SC antigens, S-100 (cytoplasmic), and p75NGFR.
‡Expression of neurofibromin (+ or −) by NF1 SC culture determined by Western blot.
§Neurofibromin immunoreactivity for originating tumor as described in Results.
¶Proliferate without GGF2.
v, varied with density; ±, heterogeneous.

to 4) cultures were maintained in standard medium and detached from the culture dish with 0.5 mmol/L ethylenediaminetetraacetic acid in phosphate-buffered saline (PBS). To examine anchorage dependency, cells were seeded in poly(HEMA)-coated culture wells at a density of 10^5 cells/well in Joklik medium (to minimize cell aggregation) supplemented with 10% serum. To examine serum dependency, cells were seeded in culture wells coated with laminin-1 (10 μg/ml) at a density of 10^5 cells/well and grown in serum-free N2 medium supplemented with 1% heat-inactivated bovine serum albumin. Cell viability was assessed at 0, 24, and 72 hours using a Trypan blue dye-exclusion assay. Counting chambers of a hemocytometer were filled with the cell suspension and viable cells (dye-excluding) as well as nonviable cells (dye-absorbing) were counted.

Growth in Soft Agarose

Anchorage-independent colony formation was determined by growing cells in soft agarose as described by Neugut and Weinstein.[25] A thin base-layer of 0.9% agarose was allowed to solidify for 1 hour in culture wells. A single-cell suspension in standard medium containing 0.4% agarose was layered over the solid agarose base and allowed to solidify. The cultures were grown for 2 to 8 weeks and viable colonies consisting of >25 cells were scored by phase-contrast microscopy. Percent colony formation was calculated as: (number of viable colonies/total viable cells seeded) × 100%.

Subcutaneous Engraftment

All animal procedures were performed in accordance with approved IACUC protocols. NF1 SC cultures were tested for their ability to form tumors after subcutaneous injection in immunodeficient *nude* mice. Cells grown on laminin in medium containing GGF-2 were harvested by trypsinization and resuspended in Hanks' balanced salt solution. Numerous subcutaneous injections were made using 2×10^6 cells/site. Mice were examined for development of palpable tumors for at least 3 months. Thereafter, animals were euthanized and the injection sites were surgically exposed and examined for signs of tumor formation. Because there were no signs of tumor growth no histology was performed.

Nerve Engraftment

Human neurofibroma-derived SC cultures (Table 1) from cryopreserved stocks were grown on laminin for 4 days in medium supplemented with GGF-2. Dissociated cells were collected, rinsed thoroughly, and resuspended as a dense slurry in Hanks' solution. Young adult *scid* mice were anesthetized and sciatic nerves of both legs were exposed at mid-thigh. A cell suspension (5×10^5 in 4 μl) was gradually injected intrafascicularly in both nerves through a fine needle (35 gauge) attached to a Hamilton syringe. The site was closed in layers with sutures and the revived mouse returned to specific pathogen-free housing. At 1 to 8 weeks after implantation, the animals were sacrificed under anesthesia and the nerves (n = 4

for each culture) were removed and fixed by immersion in 4% paraformaldehyde. Nerve segments were embedded in paraffin and sectioned for immunohistochemical staining.

Immunohistochemistry

Neurofibroma Cultures

Monolayer cultures were examined for immunoreactivity with antibodies to the SC antigens S-100 (DAKO, Carpinteria, CA) (1/300) and the low-affinity nerve growth factor receptor (p75) (4 μg/ml, hybridoma 200-3-G6-4; American Tissue Culture Collection, Rockville, MD). Cultures grown on laminin-coated chamber slides were fixed with 2% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.2) for 20 minutes, then washed with PBS containing 0.5% Triton X-100. Nonspecific antibody binding was blocked with PBS containing 0.1% Triton and 10% normal serum (blocking buffer) for 1 hour. Primary antibodies were diluted in blocking buffer and applied to wells for 2 to 4 hours at 37°C. Bound antibodies were labeled with peroxidase-conjugated secondary antibodies for 1 hour at 37°C and chromogenic development was accomplished with 3,3′-diaminobenzidine-$(HCl)_4$ (0.05%) and hydrogen peroxide (0.03%) in PBS. Bromodeoxyuridine (BrdU) incorporation *in vitro* and immunolabeling of BrdU-DNA were performed as described previously.[26]

Nerve Grafts

Sciatic nerves engrafted with neurofibroma-derived SC cultures were fixed by immersion in 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.2), sectioned in paraffin, and stained with hematoxylin and eosin for routine light microscopic examination. To identify transplanted human neurofibroma-derived SCs, nerve sections were immunostained with polyclonal anti-GSTπ (DAKO) (1/100) (a human-specific antiserum to the ubiquitous cellular protein, glutathione *S*-transferase) and a monoclonal antibody to p75 (4 μg/ml, hybridoma 200-3-G6-4) (a primate-specific antibody to the low-affinity nerve growth factor receptor). Deparaffinized sections were pretreated with methanol containing 1% hydrogen peroxide for 30 minutes to quench endogenous peroxidase activity. Nonspecific antibody binding was blocked with 10% normal serum in PBS containing 0.3% Triton X-100 for 60 minutes at 37°C. Primary antibodies were diluted in blocking buffer and applied to sections overnight at 4°C. Bound antibodies were labeled with biotinylated secondary antibodies for 4 hours at 37°C followed by the avidin-biotin-peroxidase reagent (DAKO) for 2 hours. Chromogenic development was accomplished with 3,3′-diaminobenzidine-$(HC1)_4$ (0.05%) and hydrogen peroxide (0.03%) in PBS.

Neurofibroma Tissue Specimens

Portions of the primary tumor used for cell culture were fixed by immersion in 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.2), sectioned in paraffin, and stained with hematoxylin and eosin for routine light microscopic examination. Sections were immunostained for neurofibromin with the NF1GRP(N) antibody (1 μg/ml) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) raised against a peptide corresponding to residues 509 to 528 of the predicted *NF1* gene product. The specificity of antibody to this neurofibromin peptide was reported previously.[27] Serial sections were immunolabeled with polyclonal anti-S-100 (1:300, DAKO). Immunoperoxidase labeling with the avidin-biotin-peroxidase reagent was performed as described above, except to enhance neurofibromin staining, antigen retrieval was achieved by pretreating sections in 0.1% trypsin for 20 minutes at 37°C. Immunostained sections were lightly counterstained with hematoxylin. Negative controls used no primary antibody. Additionally, for the NF1GRP(N) antibody, preadsorption with a 10-fold molar excess of peptide antigen (SC-67P, Santa Cruz) was used to achieve complete blocking of neurofibromin immunoreactivity.

Western Immunoblotting

SC cultures were scraped from dishes and cell pellets were homogenized in ice-cold extraction buffer consisting of 50 mmol/L Tris-HCl (pH 7.4), 250 mmol/L NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, and complete protease inhibitors (Boehringer-Mannheim, Indianapolis, IN). The soluble fraction was collected by centrifugation (10,000 $\times$ *g*, 20 minutes) and then was made 2 mol/L in urea. The extract was concentrated and fractionated by ultrafiltration using a 100-kd cut-off membrane. Total protein content of the high molecular mass retentate was determined using Bradford Reagent (Bio-Rad Laboratories, Hercules, CA). Samples were mixed with sodium dodecyl sulfate-containing electrophoresis sample buffer containing 2 mol/L urea and 5% 2-ME and then heated to 80°C for 2 hours. Samples (100 μg) were electrophoresed on 4 to 15% polyacrylamide gels and electroblotted to nitrocellulose sheets in transfer buffer containing 0.1% sodium dodecyl sulfate. Blots were rinsed in water and fixed in 25% isopropanol/10% acetic acid. Nitrocellulose sheets were washed with 0.05 mol/L Tris-HCl (pH 7.4) containing 1.5% NaCl and 0.1% Triton X-100 and then blocked in the same buffer with the addition of 5% dry milk (blocking buffer). The blots were incubated for 2 hours with anti-NF1GRP(N) antibody (1 μg/ml) in blocking buffer. Bound antibody was detected by peroxidase conjugated swine anti-rabbit IgG (affinity purified, DAKO) diluted 1/2,000 in blocking buffer. Immunoreactive bands were developed by chemiluminescent methods (Pierce Chemical Co., Rockford, IL) according to the manufacturer's instructions. Relative molecular mass was determined using prestained markers including myosin (205 kd). Control samples were similarly processed from cell pellets obtained from normal human nerve SC cultures and SC cultures derived from embryonic homozygous *Nf1* knockout mice.[12]

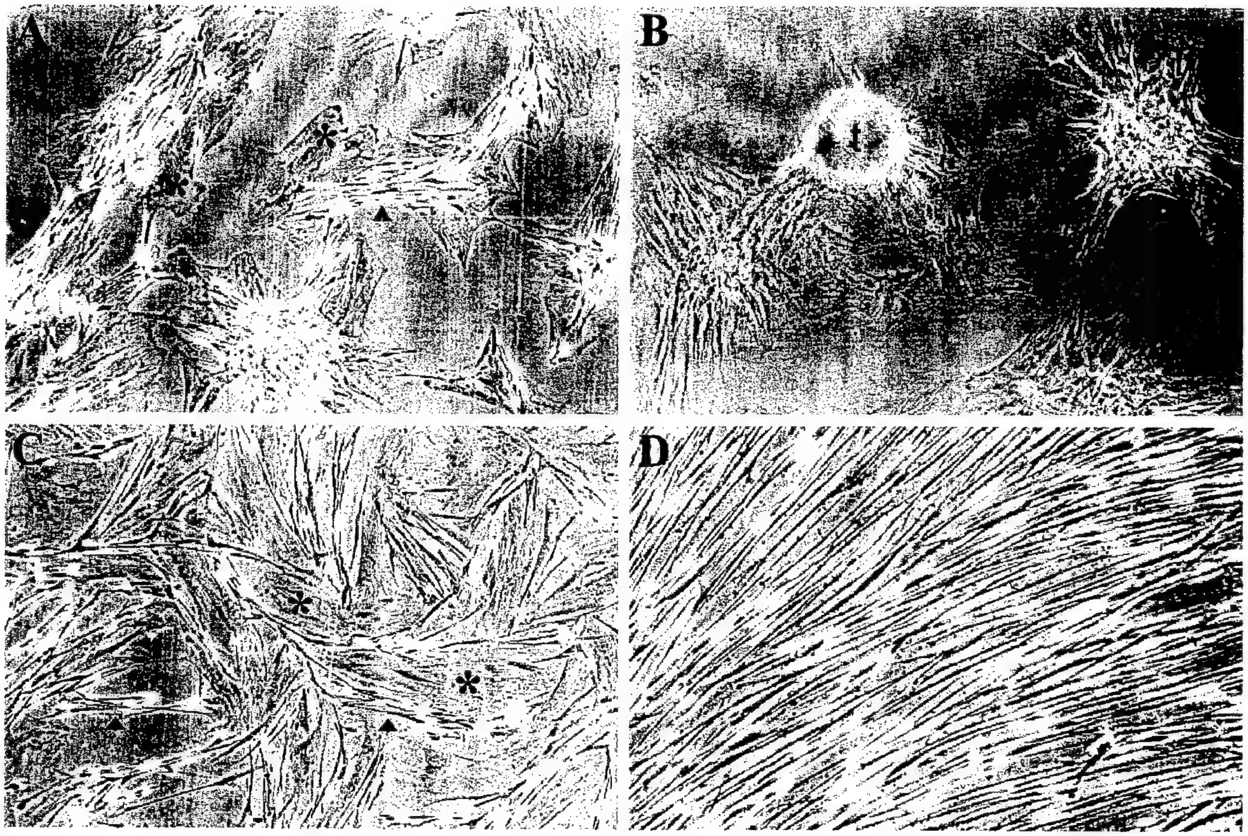

Figure 1. Subculture of neurofibroma-derived SCs. **A:** A typical primary neurofibroma culture containing mainly SLCs and FLCs. Seeded on tissue culture plastic, phase-bright SLCs (**arrowhead**) grew exclusively on patches of underlying FLCs (**asterisk**). **B:** SLCs proliferated in response to GGF-2 and formed dense cell aggregates (culture tumors, t) demonstrating their lack of contact-inhibited grow and continued association with the FLC sublayer. **C:** SLCs preferentially attached to a laminin substratum, which circumvented the association of SLC to the FLC layer and allowed expansion of the SLCs. **D:** A highly enriched SLC population was subcultured by growth on laminin in the presence of GGF-2 within three to four passages. Original magnification, ×200.

Results

Culture of Normal Human Nerve SCs

SCs do not proliferate in response to standard serum-supplemented medium and, until recently, methods for isolating normal human SCs were mostly unreliable. Based on recent advances,[20,21] we investigated various means to enrich and expand SCs from adult human nerve segments. Successful enrichment of SC cultures and depletion of fibroblastic cells was readily achieved by treatment with the SC mitogens GGF-2 and forskolin combined with differential cell detachment and preferential growth on a laminin-coated substratum. After 3 to 4 passages under these conditions, hundreds of millions of SCs were obtained nearly free of fibroblast contamination from several centimeters of adult tibial nerve. These cultures were homogeneous and contained highly elongated SCs that stained intensely for S-100 and p75, recognized markers for cells committed to a SC lineage.[28] The specificity of S-100 as a marker for cultured human SCs is shown in Figure 3A. After enrichment and expansion, SC division rapidly decreased in the absence of GGF-2 and forskolin. Growth on a laminin substratum was required at all stages to improve attachment and to minimize cell attrition. SC expansion was limited to ~10 population doublings before senescence regardless of mitogen stimulation, confirming the earlier findings by Rutkowski and colleagues.[29]

Culture of Neurofibroma SCs

Our goal was the enrichment and characterization of SLCs from dermal and plexiform neurofibromas. Monolayer cultures of neurofibromas were initiated by enzyme dissociation under standard culture conditions. The most frequent primary culture obtained from dermal and plexiform neurofibromas contained a sublayer of fibroblast-like cells (FLCs) admixed with numerous (20 to 60%) spindle-shaped SLCs (Figure 1). Numerous procedures to enrich and expand SLCs from the primary cultures were tested. It is notable that enrichment of SLCs was not improved by combined treatment with GGF-2 and agents that elevate cAMP. In particular, forskolin caused considerable heterogeneity in the SC population and hampered the development of the SLC cultures described below. Thus, unlike normal SC cultures, neurofibroma cultures were treated only with GGF-2 and were not exposed to forskolin.

Categorical responses to SC enrichment procedures (see Materials and Methods) emerged for the neurofibroma cultures. We defined four culture types based on prevalent cell morphologies, growth under standard cul-

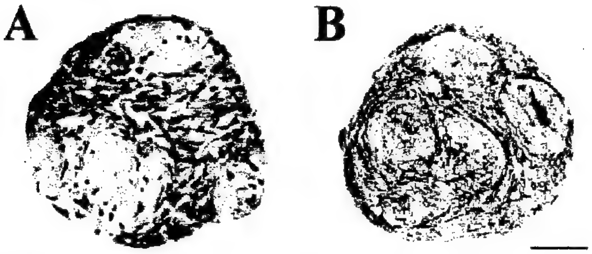

Figure 2. Neurofibroma SCs aggregated and formed culture tumors. Aggregates of SLCs, such as those shown in Figure 1B, were collected from early neurofibroma cultures and sectioned. **A:** H&E staining of culture tumors revealed areas of high cell density and cell-sparse regions of dense extracellular matrix similar to those seen in neurofibromas. **B:** Immunolabeling with laminin demonstrated a rich basement membrane network throughout the SC aggregates. Scale bar, 50 μm.

ture conditions, growth response to GGF-2 and laminin, and the onset of senescence. Findings were based on 40 robust cultures, 24 from dermal and 16 from plexiform neurofibromas.

A first type of neurofibroma culture (type-1) was distinguished by a poor response to the SC enrichment treatment with GGF-2 and laminin. These cultures (from dermal and plexiform tumors) were rapidly dominated by FLCs that often displayed abnormal growth characteristics (eg, rapid and protracted proliferation and loss of density-limited growth). The SLCs (immunopositive for S-100 and p75) failed to proliferate sufficiently and became increasingly diluted with repeated passage. Type-1 cultures accounted for 14 of 24 dermal and eight of 16 plexiform cultures, were deemed intractable to SLC enrichment, and were excluded from the analyses to follow.

A second subset, type-2 neurofibroma cultures, were amenable to enrichment of the SLCs. In most of these cultures the SLCs proliferated rapidly in the presence of GGF-2 and soon formed confluent islands on top of underlying FLCs (Figure 1A). Cultured on native tissue culture plastic, the SLCs attached exclusively to the FLCs. Despite the limited surface area of the underlying the FLCs, proliferation by the SLCs continued in the presence of GGF-2. As a result, the SLCs formed dense cellular masses or culture tumors (Figures 1B and 2A), indicating the absence of contact inhibition (density-limited growth) by neurofibroma SLCs. Immunolabeled sections of these culture tumors showed an extensive laminin-rich extracellular matrix (Figure 2B). These observations potentially relate to the adhesive mechanisms involved in the growth and development of neurofibromas.[30]

Growth on a laminin substratum, which circumvented the adhesion of the SLCs to the FLCs, was necessary for the subsequent enrichment and expansion of the SLCs (Figure 1C). Once outnumbered by the mitogen-driven SLCs, the FLCs were effectively diminished by differential detachment. Thereafter, the SLC population was readily enriched and expanded in large numbers within 3 to 4 passages (Figure 1D). Type-2 SLC cultures stained for S-100 and p75 and generally resembled highly spindled SCs obtained from normal nerve (Figure 3, A and B). However, purified SLCs from some neurofibromas, particularly those from plexiform tumors, were stubby and less elongated (Figure 3C). The SLCs derived from dermal neurofibromas had a limited proliferative capacity similar to that of normal adult SCs (10 doublings) and became senescent thereafter. By comparison, the plexiform SLCs were less restricted and often were passaged >20 times before showing signs of senescence. All type-2 SLC-enriched neurofibroma cultures grew very slowly when withdrawn from GGF-2, but were stable for months on a laminin substratum. SLC-enriched type-2 cultures accounted for eight of 24 dermal and two of 16 plexiform cultures.

A third type of neurofibroma culture was obtained exclusively from plexiform tumors and contained SLCs with several preneoplastic properties. Type-3 SLCs expanded rapidly without close association with the FLC sublayer and were easily enriched to near homogeneity. Five type-3 cultures were established from plexiform tumors; SLCs in two cultures had a stubby, spindle shape whereas the other three cultures were multipolar or polygonal (Figure 3D). In each culture, nearly all cells were stained for S-100, whereas p75 expression varied. Three multipolar/polygonal type-3 cultures grew particularly well (doubling times 2 to 4 days) in response to serum and GGF did not further increase their growth rates. These cultures also showed protracted expansion (>20 to 50 passages). The occurrence of these preneoplastic properties is almost certainly indicative of genetic abnormalities originating *in vivo* because growth factor-independent proliferation and expansion beyond 10 passages were never observed in cultures of normal human SCs (also see Rutkowski et al[29]). Furthermore, the three GGF-independent type-3 cultures were derived from sizable recurrent plexiform neurofibromas.

A fourth subset of neurofibroma culture, at low density, were pleomorphic, lacy and phase-pale, and stained faintly, or not at all, for S-100 and p75. However, with increasing density they became elongated spindle cells and grew in parallel arrays. Remarkably, when grown to confluency, these cultures formed dense ridges of S-100-expressing SLCs (not shown). The appearance of these S-100-positive SLCs did not decrease after numerous passages but, instead, continued to increase in number with increasing culture density. These cultures were expanded extensively (>50 passages) before showing signs of senescence. These observations suggest that these cultures contained a poorly differentiated cell type capable of giving rise to a reversibly differentiated SLC component. This type of pleomorphic property also was observed in several neurofibrosarcoma cultures (not shown).

In summary, 40 primary cultures were established from 24 dermal and 16 plexiform neurofibromas and subcultured for enrichment of SLCs. Twenty-two of 40 cultures did not respond to SLC enrichment or were predominantly fibroblastic (type-1). Ten dermal and eight plexiform tumor cultures yielded enriched SLC cultures (type-2, -3, and -4) suitable for additional genetic and biological studies (listed in Table 1). The type-2 and type-3 cultures (eight from dermal and seven from plexiform tumors) were the focus of subsequent studies.

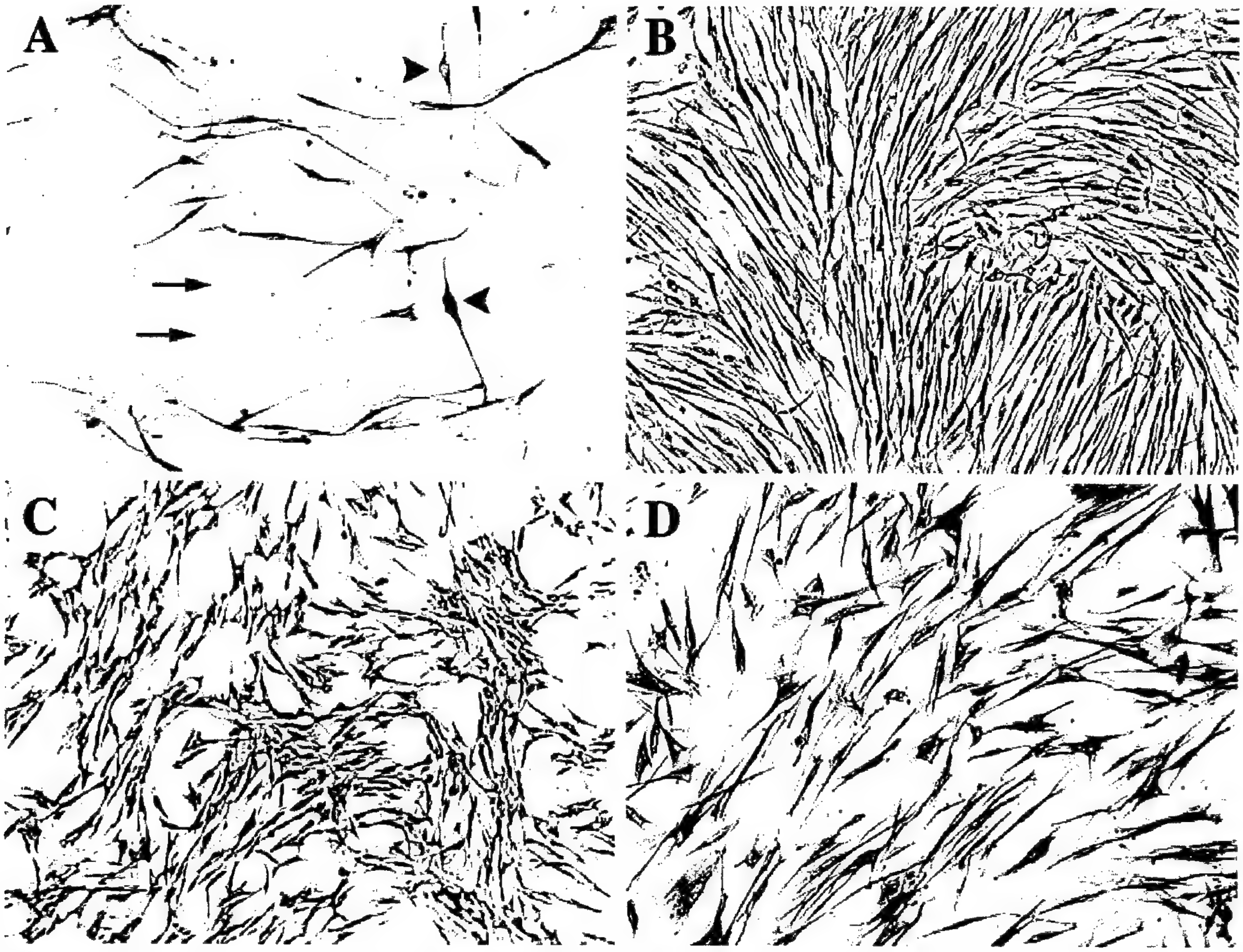

Figure 3. Morphology and S-100 immunostaining of nerve and neurofibroma-derived SC cultures. **A:** A primary culture established from normal adult human nerve, like those from neurofibromas, contained a mixture of S-100-positive SCs (**arrowheads**) and fibroblastic cells (**arrows**). SCs showed intense cytoplasmic S-100 immunoreactivity, while faint (if any) staining was visible in the nuclei of fibroblastic cells (contrast-enhanced image). After enrichment and expansion of SLCs from neurofibromas, the resulting SC cultures immunostained positive for S-100 yet often differed in morphology and growth properties (**B–D**). **B:** Type-2 cultures from dermal neurofibromas most often showed highly elongated spindle-shape cells similar to normal SCs. **C:** Type-2 and some type-3 cultures from plexiform neurofibromas generally contained multipolar and stubby spindled cells. **D:** Type-3 cultures were obtained exclusively from plexiform tumors and contained elongated but primarily polygonal cells. Original magnification, ×200.

Neurofibromin Expression by SCs in Neurofibromas

Before neurofibroma specimens were prepared for cell culture, representative specimens of tumor were fixed and processed for routine histology and immunohistochemistry. Paraffin sections were immunostained with an anti-neurofibromin peptide antiserum. Neurofibromin immunoreactivity in normal control tissues was similar to that reported by other laboratories.[31] In neurofibromas the SC elements were identified by their nuclear characteristics (elongated, wavy nuclei with pointed ends) and positive immunoreactivity for S-100 protein (Figure 4). Four basic patterns of neurofibromin immunoexpression in S-100-positive regions were observed: 1) neurofibromin-negative ($-/^-$); 2) predominantly neurofibromin-negative with focal areas of distinct positive staining ($-/^+$); 3) predominantly immunoreactive tumor with focal areas of distinct negativity ($+/^-$); and 4) neurofibromin-positive ($+/^+$). Immunoreactivity patterns for the surgical resection specimens corresponding to the SC cultures from which they were derived are summarized in Table 1. The majority (14 of 17) of the neurofibromas that gave rise to SC cultures were predominately neurofibromin-negative (designated $-/^-$ and $-/^+$ in Table 1). Moreover, more than one-half of these tumors were completely negative ($-/^-$), showing no neurofibromin immunostaining in any SC elements (Figure 4A). Furthermore, even in the few predominately neurofibromin-positive tumors ($+/^-$), many individual SCs were negative for neurofibromin (Figure 4C). In areas of positive immunoreactivity, tumor cells showed discrete, granular staining in the perinuclear cytoplasm and in delicate elongated processes. In tumors with positively and negatively stained regions, the regions of neurofibromin nonreactivity were histologically similar to immunoreactive areas. Overall, neurofibromin immunoexpression in most neurofibromas that gave rise to a SC culture was very low (see Discussion). The finding of both positive and negative areas of neurofibromin immunoreactivity in some neurofibromas could not be at-

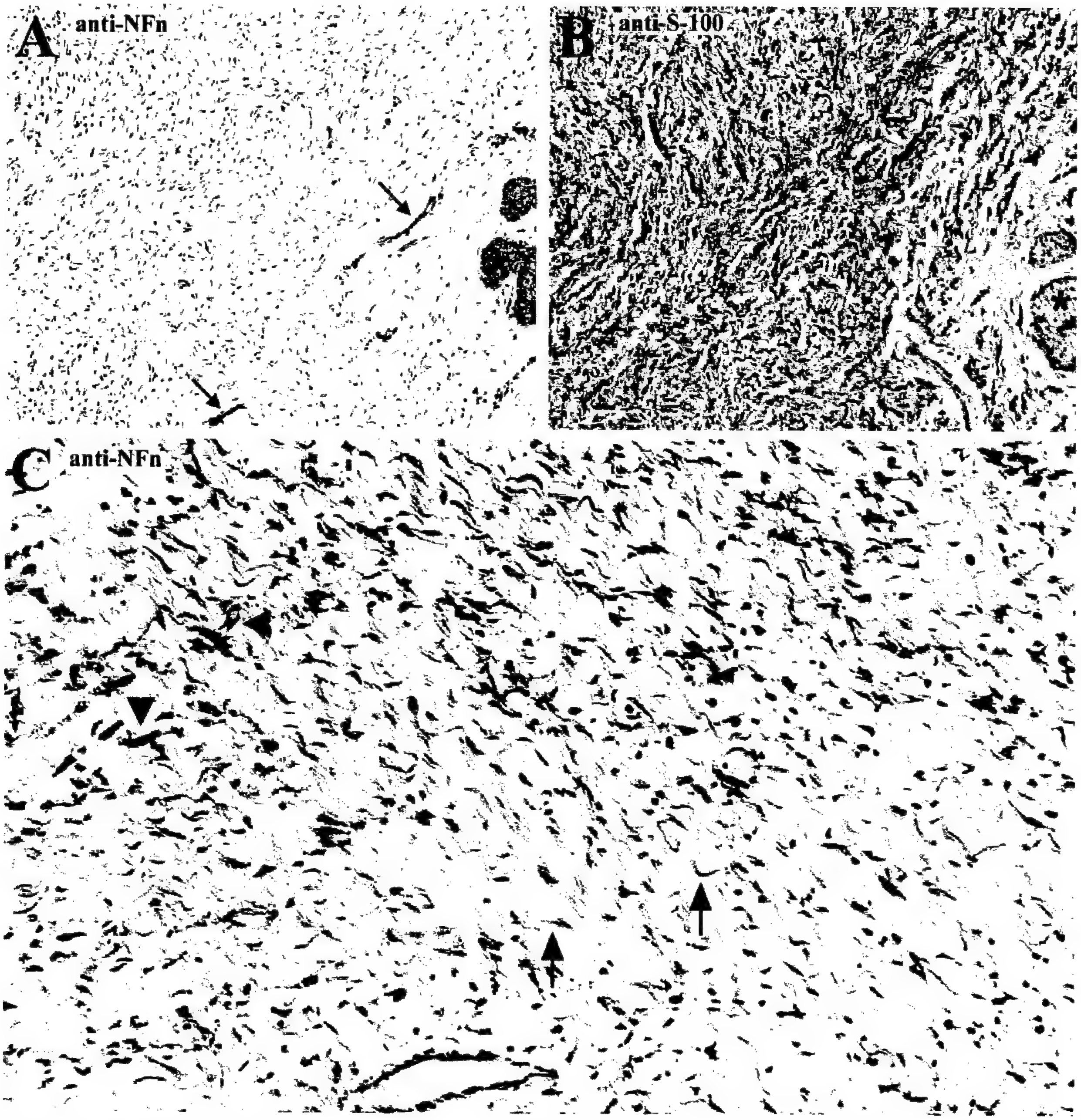

Figure 4. Neurofibromin immunoreactivity of SCs in neurofibromas. Tumor sections were immunoperoxidase stained for neurofibromin (**A** and **C**) and S-100 (**B**). **A:** Most neurofibromas that gave rise to SC cultures were either mainly or completely devoid of neurofibromin-positive SCs. Vascular elements (**arrows**) and sweat glands (**asterisk**) stained for neurofibromin and served as internal positive controls. **B:** A serial section of the neurofibroma shown in **A** showing strong and diffuse immunoreactive for the SC marker protein, S-100. **C:** A neurofibroma containing intermixed populations of neurofibromin-positive (**arrowheads**) and neurofibromin-negative SCs (**arrows**). In areas of positive immunoreactivity, tumor cells showed discrete, granular staining in the perinuclear cytoplasm and in delicate elongated processes. SC elements were identified by their nuclear characteristics (elongated, wavy nuclei with pointed ends) and positive immunoreactivity for S-100 protein. Sections were counterstained with hematoxylin. Original magnifications: ×200 (**A** and **B**), ×400 (**C**).

tributed to artifacts of fixation, tissue preparation, or regional differences in antibody concentration. Dermal and vascular elements were uniformly positive for neurofibromin in both immunoreactive and nonreactive regions of a given tumor (Figure 4). Also, tumors that were negative or that contained areas of neurofibromin nonreactivity were all strongly and widely immunoreactive for S-100 protein (Figure 4B).

Neurofibromin Expression by NF1 SC Cultures

We hypothesized that the abnormal growth properties of NF1 SC cultures, particularly the type-3 cultures, are the result of a severe deficiency in neurofibromin expression. Normal SCs and type-2, -3, and -4 NF1 SC cultures were examined for the expression of neurofibromin by Western immunoblotting. Results are shown in Figure 5. Antibody

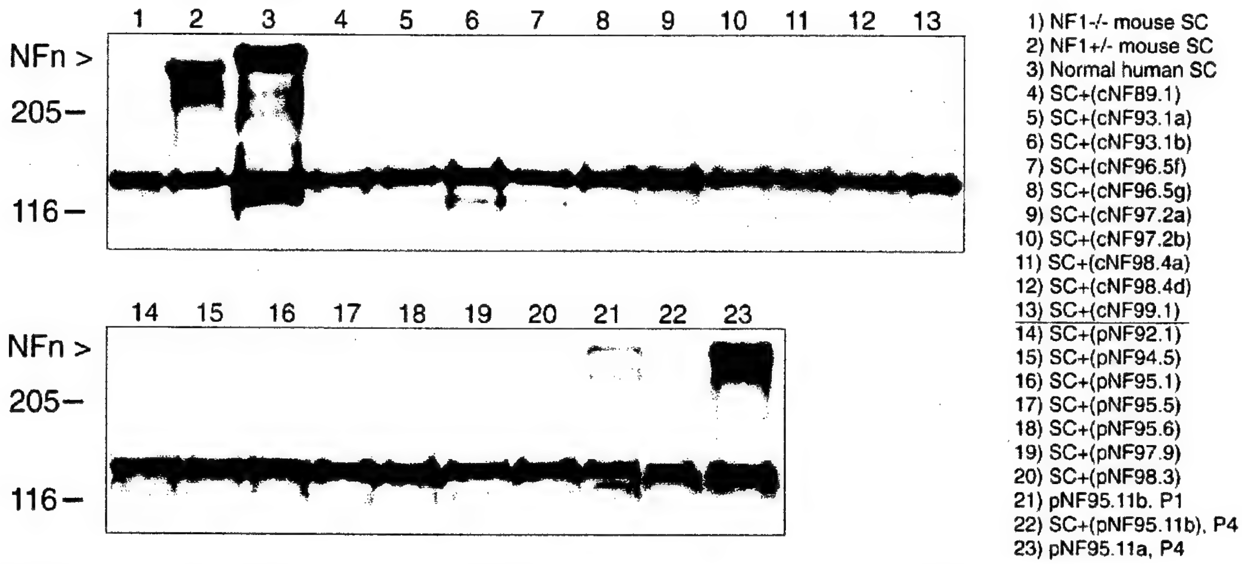

Figure 5. Western immunoblot analysis of the SC cultures for neurofibromin expression. Culture cell pellets were extracted and a high molecular mass fraction was obtained by ultrafiltration (>100 kd). Samples (100 μg of total protein) were electrophoresed and transferred to nitrocellulose sheets. The blots were stained for neurofibromin using antibody NF1GRP(N) and chemiluminescent detection. Antibody specificity was demonstrated on extracts of SC-enriched cultures from embryonic *Nf1* knockout mice. Full-length neurofibromin was absent from homozygous cultures (*Nf1*$^{-/-}$) (**lane 1**), whereas heterozygous cultures (*Nf1*$^{+/-}$) (**lane 2**) expressed a predominant immunoreactive band-pair with a M_r = 225 to 250 kd, slightly smaller than the M_r = 240 to 260 kd neurofibromin bands produced by normal human SCs (**lane 3**). A band at 140 kd appeared in each sample that, although undefined, indicated the consistency of total protein loaded in each lane. Full-length neurofibromin was absent in extracts of the 10 dermal SC cultures (**lanes 4** to **13**) and eight plexiform SC cultures (**lanes 14** to **20** and **22**). Also shown is the original mixed primary culture pNF95.11b (**lane 21**) from which the type-3 SC culture, SC+(pNF95.11b), was derived (**lane 22**) and the type-1 culture, pNF95.11a (an earlier specimen from the same patient).

specificity was examined on extracts of SC-enriched cultures from embryonic *Nf1* knockout mice. Full-length neurofibromin was absent from homozygous cultures (*Nf1*$^{-/-}$) (lane 1), whereas heterozygous cultures (*Nf1*$^{+/-}$) (lane 2) expressed a predominant immunoreactive band-pair with a M_r = 225 to 250 kd (slightly smaller than that produced by human cells). A band at 140 kd appeared in each sample that, although undefined, indicated the consistency of total protein loaded in each lane. Extracts of normal human SC cultures (lane 3) contained a predominant neurofibromin-immunoreactive band-pair with a M_r = 240 to 260 kd. In contrast, full-length neurofibromin was absent in extracts of the 10 dermal SC cultures (lanes 4 to 13) and eight plexiform SC cultures (lanes 14 to 20 and 22). As an example, we also show the mixed primary culture pNF95.11b (lane 21) from which the SC culture, SC+(pNF95.11b) (lane 22), was derived. The first passage of the culture pNF95.11b contained an admixture of cells (including numerous FLCs and perhaps diverse SC lineages). A modest band-pair corresponding to full-length neurofibromin was observed in this culture extract (lane 21), indicating the genetic heterogeneity in this early culture. This finding is consistent with the pattern of neurofibromin immunostaining (+/$^-$) observed in the originating tumor sections (Table 1). However, neurofibromin expression was undetectable in the derived type-3 SC culture (lane 22). The pNF95.11b cultures were established from a resection of a recurrent plexiform neurofibroma. Two years earlier, we established a culture from a specimen obtained from the initial resection. This culture, pNF95.11a, was type-1 (dominated by FLCs and intractable to SC enrichment) and expressed abundant neurofibromin (lane 23). Neurofibromin content in this culture extract was presumably contributed by the large population of fibroblasts, but we cannot exclude the contribution by *NF1* heterozygous SCs. From these results we conclude that neurofibromin-deficient SCs have the best long-term growth advantages and that our subculture methods to enrich for SCs from neurofibromas are highly selective for neurofibromin-deficient SCs.

Properties of NF1 SC Cultures in Classical Tumorigenic Assays

Neoplastic and tumorigenic properties of type-2 and -3 neurofibroma SC cultures were examined in several classical tests, including assays of serum and anchorage dependence, colony formation in soft agarose, and subcutaneous tumor formation in immunodeficient mice. The proliferative properties (normal and abnormal) of these cultures were described in a previous section. Although not all of the NF1 SC cultures listed in Table 1 were tested repeatedly, findings of tumorigenic properties were consistent for all type-2 and -3 cultures from dermal and plexiform tumors. Notably, all type-2 and type-3 NF1 SC cultures showed properties similar to normal human SCs. First, the survival of the NF1 SC cultures and normal SCs was not highly growth factor-dependent, as 85 to 95% of cells remained viable in serum-free medium for at least 72 hours (compared to 95% survival of the highly tumorigenic RN22 schwannoma and C6 glioma cell lines). Proliferation was not observed in any of the SC cultures in the absence of serum. Second, the survival of the SC cultures was anchorage-dependent. Under nonadherent

conditions the percentage of viable normal SCs dropped below 40% after 72 hours. Similarly the survival of NF1 SC cultures was 20 to 40% after 72 hours in suspension culture (compared to >95% in the RN22 and C6 lines).

Colony formation in soft agarose is an indicator of high tumorigenicity and anchorage-independent growth, properties of transformed cells and some, but not all solid tumor cultures. Suspended as single cells in soft agarose, 72% of RN22 and 88% of C6 cells proliferated and rapidly formed colonies that become visible to the eye within 2 weeks. In contrast, multicellular foci of normal SC or NF1 SC cultures were rarely observed and none reached the 25-cell colony criterion, even after cultured for 2 months in agarose.

NF1 SC cultures showed no subcutaneous tumorigenic growth. Millions of cells per site were injected subcutaneously in *nude* mice. No palpable tumors were found after 3 months and there were no subcutaneous foci visible in postmortem examinations. Taken together, these observations indicate that the neurofibroma SC cultures, even the type-3 cultures, had low tumorigenic potential in these classical assays.

Neurofibroma SC Cultures Grafted in the Mouse Nerve

The tumorigenic growth of selected neurofibroma SC cultures was examined as xenografts in the sciatic nerves of adult immunodeficient *scid* mice. Each NF1 culture was engrafted into four nerves and six nerves were engrafted with an equal number of normal human SCs. Engrafted nerves were examined by immunostaining with an antibody specific to human glutathione *S*-transferase (Figure 6). First, transplantation of normal SCs resulted in transient occupancy (Figure 6C) and survival appeared to be severely limited because in four of six of the nerves glutathione *S*-transferase labeling was absent after 8 weeks. In contrast, all neurofibroma SC cultures (seven of seven) showed persistent and diffuse intraneural growth throughout the same period. Typically, neurofibroma SCs emanated from glutathione *S*-transferase-positive foci and grew in extensive longitudinal streams that intermingled with the host nerve elements (Figure 6A). Tumor cell migration also was associated with the nerve sheaths. Some NF1 SC cultures developed sizable masses that displaced nerve elements and caused significant regional enlargement of the nerve diameter (Figure 6B). Tumor masses varied in size for the different culture grafts, but additional work is required to quantitate the size and distribution of the tumor grafts. It will also be important to determine whether growth rates of the cultures, grafts, and originating tumors are related. Nevertheless, all observations indicated that tumor development in the mouse nerve, like that of human neurofibromas, was relatively slow and benign. There were only sporadic signs of functional impairment associated with the largest tumors and no mortality was associated with the transplants for up to 8 weeks. These results demonstrate reliable and sustained tumor growth by neurofibroma-derived human SCs implanted in the mouse nerve.

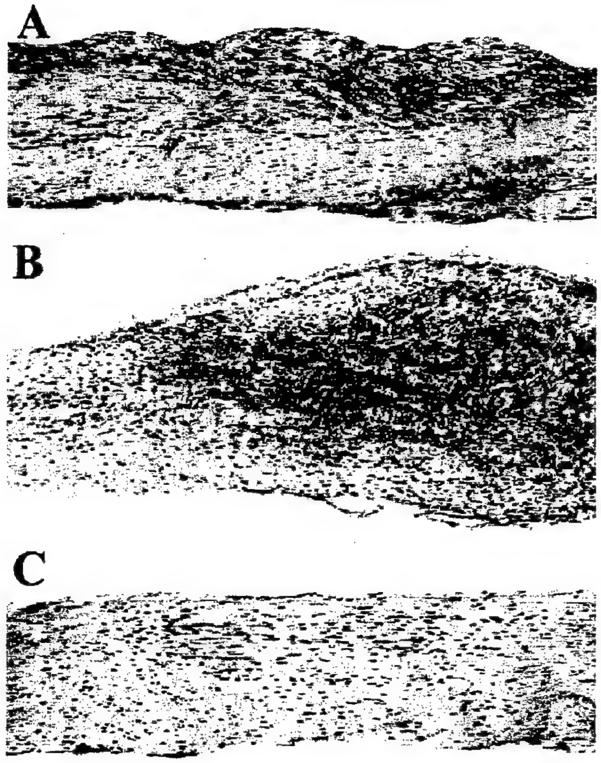

Figure 6. Tumor growth by human NF1 SC cultures xenografted into the nerves of *scid* mice. SC cultures from neurofibromas were transplanted into the nerves of immunodeficient mice. The growth and distribution of engrafted cells was traced by immunostaining with an antibody specific to human glutathione *S*-transferase. NF1 SC xenografts 4 weeks (**A**) and 8 weeks (**B**) after engraftment in the mouse sciatic nerve developed sizable masses and substantially increased the nerve diameter. NF1 SCs invaded along the longitudinal nerve axis between axons and along the epineurial sheath. Even the most extensive graft of normal SCs showed meager growth after 8 weeks, whereas most normal SC grafts failed to survive (**C**). Sections were counterstained with hematoxylin. Original magnification, ×100.

Discussion

In general, neurofibroma SCs, like their counterparts in normal nerves, fail to proliferate *in vitro* in the absence of specific mitogens.[5,7] Past efforts to establish models for human neurofibromas have relied on tissue explants and primary cultures of limited cell number with marked cellular heterogeneity. Only very recently have SCs been isolated from neurofibromas.[17–19] The lack of human neurofibroma-derived SC cultures and the low tumorigenic potential of neurofibroma cells in animal models has hampered the study of these prevalent tumors. Here we report the subculture of SCs from 10 dermal and eight plexiform NF1 tumors. All 18 of these NF1 SC cultures were found to be neurofibromin-deficient. Interestingly, many of the originating tumors contained both neurofibromin-negative and neurofibromin-positive SCs, indicating that SCs that lack functional *NF1* alleles have a distinct growth advantage *in vitro*. This advantage has been exploited by our subculture enrichment procedures using GGF-2 (without forskolin) and laminin.

Loss of *NF1* gene expression has been reported in malignant and benign SC tumors and pheochromocytomas from patients with NF1.[32,33] Based on the two-hit

hypothesis of tumor suppressor genes such as *NF1*, it is expected that neurofibromas should contain a supernumerary population of neurofibromin-deficient cells that are tumorigenic. Recent findings of cytogenetic alterations, loss of heterozygosity, and the absence of *NF1* mRNA expression in SCs cultured from neurofibromas strongly implicate SCs as the major neoplastic elements of dermal and plexiform neurofibromas.[17–19] In addition, in the present study we found that SCs isolated from numerous neurofibromas lack neurofibromin expression, confirming that *NF1* is inactivated in these SCs. Despite these persuasive findings, immunohistochemical evidence for a dominant population of neurofibromin-deficient cells has been highly ambiguous and scarcely reported. In an earlier study of numerous NF1 tumors, we concluded that a majority of neurofibromas consist mainly of SCs that express neurofibromin.[34] This antithetical observation was based on a large number of random archival specimens and, moreover, held true for a significant proportion of neurofibrosarcomas as well. However, on closer scrutiny, to examine the two-hit hypothesis at the single-cell level, it became evident that neurofibromin-negative SCs were present in most, if not all, neurofibromas. In the present study we focused on an independent group of neurofibromas from which SC subculture was successful. In most of the tumors in this group, neurofibromin labeling of tumoral elements was particularly sparse or absent. The remainder contained mixed populations of neurofibromin-negative and neurofibromin-positive SCs. The neurofibromin antibody used in these studies was raised against a peptide corresponding to amino acids 509 to 528 of the predicted *NF1* gene product. Directed against an N-terminal epitope, the antibody should bind to known neurofibromin splice variants as well as highly truncated (abnormal) forms. These results provide strong evidence that neurofibromin-negative cells of SC lineage contribute centrally to the formation of at least a significant subset of both dermal and plexiform neurofibromas. Nevertheless, there was notable variability in neurofibromin expression by SCs within many of the neurofibromas. The presence of neurofibromin expressing (*NF1* heterozygous) SCs suggests they too may perpetuate tumor formation, perhaps driven by the paracrine influence of the neurofibromin-deficient cell population. In this regard, Gutmann and co-workers[35] reported that neurofibromin expression by SCs in benign tumors may be down-regulated by factors produced within the tumor. Thus, paracrine influences may represent a novel mechanism for inactivating growth-suppressing genes and allowing for increased cell proliferation in tumors even in nonclonal cells. It is interesting that proliferation of neurofibromin-deficient SCs in response to GGF-2 was not enhanced by forskolin. Because forskolin increases SC expression of growth factor receptors including the GGF receptors erbB2 and erbB3,[36,37] this suggests that neurofibroma SCs express high levels of GGF receptors. Hyperexpression of erbB receptors has been reported in NF1 tumors and an inverse expression pattern of erbB2 and neurofibromin was shown for human SCs.[38,39] Additionally, SCs can express GGF and, at doses submaximal for proliferation, GGF-2 increases and directs the migration of SCs.[40–42] Taken together, these findings raise the possibility that GGF may function in an autocrine/paracrine mechanism that supports the continued growth of SCs in neurofibromas.

Despite considerable advances in the molecular genetics of NF1, the histogenesis of neurofibromas remains enigmatic. Dermal and plexiform neurofibromas contain a variety of cell types including SCs, perineurial cells, and fibroblasts. It is commonly held that despite their cellular complexity the histological features of neurofibromas are monotonously consistent.[43] In contradistinction, our studies of numerous neurofibromas and their derivative cell cultures indicate there are several levels of cellular and genetic diversity in this class of benign peripheral nerve sheath tumor. Cytogenetic abnormalities were identified in one of five of the dermal (our unpublished observation) and four of six of the plexiform SC cultures.[17] There were no consistent chromosomal regions involved in the abnormal karyotypes, suggesting that originating tumors are heterogeneous and may bear a variety of primary and/or secondary genetic changes. Additionally, the two plexiform cultures that displayed no cytogenetic rearrangements showed GGF-independent growth, suggesting that they either contain underlying genetic abnormalities not yet detected, or have expression abnormalities because of epigenetic influence. Clearly, neurofibromin deficiency did not confer GGF-2-independent growth on all of the developed SC cultures. Despite some differences in morphology, GGF-2 dependence, and karyotype, all NF1 SC cultures showed similarly low tumorigenic potential in several classical *in vitro* assays. However, the neurofibroma SC cultures showed a strong propensity to aggregate and form culture tumors. A similar growth pattern was observed for suspension cultures whereby NF1 SCs readily grew in aggregates reminiscent of tumor spheroids (an *in vitro* model for tumorigenic growth). Neurofibroma culture tumors contained an extensive laminin-rich extracellular matrix similar to that observed in neurofibromas.[30] These observations attest to a tumorigenic property of neurofibromin-deficient SC cultures that may be related to the adhesive mechanisms involved in the growth and development of neurofibromas. Sheela and co-workers[15] first demonstrated that NF1 SCs are angiogenic and invasive. In subsequent studies, which included two of the NF1 SC cultures used in the present report, we also concluded that NF1 SCs have a high invasive potential and a loss of negative autocrine growth control.[16] Despite these tumorigenic properties, our NF1 SC cultures, as well as the neurofibroma cultures used by Sheela and colleagues,[15] failed to form subcutaneous tumors in immunodeficient mice. Taken together, these findings imply that NF1 SCs have a tumorigenic potential that was not fully expressed in the *in vivo* model systems used previously.

Inceptive studies demonstrated the growth of implanted human neurofibroma tissue or SC preparations into the sciatic nerves of immunodeficient mice and the potential of this xenograft model for studying the tumorigenesis in NF1.[14,44] In the present study neurofibroma-like tumors resulted from the transplantation of neurofibromin-deficient NF1 SC cultures into the nerves of *scid*

mice. Extensive migration was consistently observed and many tumors were sizeable and substantially enlarged in the mouse nerve. There were apparent differences in the growth by the different transplanted NF1 SC cultures but overall tumor expansion was slow, suggesting that the growth rate of the developed tumors may reflect that of human neurofibromas. Additional studies are required to determine whether the growth patterns of the engrafted SC tumors correlate with those of the originating human tumors. This intraneural engraftment model is the first to achieve tumorigenic growth *in vivo* by human neurofibromin-deficient SCs and provides the means to study the histogenesis of neurofibromas in a relevant cellular environment. A further enhancement to this NF1 tumor model will be to transplant these neurofibroma SCs in the nerves of immunodeficient mice that are also heterozygous for *Nf1*. This highly relevant model of neurofibroma will also provide the opportunity to observe the interactions and contributions of engrafted ($NF1^{-/-}$) cells and ($Nf1^{-/+}$) nerve elements with the same genetic background as those found in NF1 patients.

Acknowledgments

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Chromosome 17 Loss-of-Heterozygosity Studies in Benign and Malignant Tumors in Neurofibromatosis Type 1

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Neurofibromatosis type 1 (NF1) is a common autosomal dominant condition characterized by benign tumor (neurofibroma) growth and increased risk of malignancy. Dermal neurofibromas, arising from superficial nerves, are primarily of cosmetic significance, whereas plexiform neurofibromas, typically larger and associated with deeply placed nerves, extend into contiguous tissues and may cause serious functional impairment. Malignant peripheral nerve sheath tumors (MPNSTs) seem to arise from plexiform neurofibromas. The *NF1* gene, on chromosome segment 17q11.2, encodes a protein that has tumor suppressor function. Loss of heterozygosity (LOH) for *NF1* has been reported in some neurofibromas and NF1 malignancies, but plexiform tumors have been poorly represented. Also, the studies did not always employ the same markers, preventing simple comparison of the frequency and extent of LOH among different tumor types. Our chromosome 17 LOH analysis in a cohort of three tumor types was positive for *NF1* allele loss in 2/15 (13%) dermal neurofibromas, 4/10 (40%) plexiform neurofibromas, and 3/5 (60%) MPNSTs. Although the region of loss varied, the p arm (including *TP53*) was lost only in malignant tumors. The losses in the plexiform tumors all included sequences distal to *NF1*. No subtle *TP53* mutations were found in any tumors. This study also reports the identification of both *NF1* "hits" in plexiform tumors, further supporting the tumor suppressor role of the *NF1* gene in this tumor type. *Genes Chromosomes Cancer 28:425–431, 2000.*

INTRODUCTION

Neurofibromatosis type 1 (NF1) is a common autosomal dominant condition, occurring in approximately 1/3,000 individuals. It is characterized primarily by neurofibromas, axillary freckling, café-au-lait spots, and Lisch nodules (Huson, 1994). Affected individuals also have an increased risk of malignancy. Dermal neurofibromas are benign lesions, primarily of cosmetic significance. These occur in most adult NF1 patients, typically developing during adolescence and adulthood, with most individuals developing dozens to thousands in their lifespan. These tumors have virtually no risk of malignant transformation. In contrast, plexiform neurofibromas usually involve deep nerves, have a complex architecture, and frequently extend into contiguous structures. They are often congenital and can cause severe functional impairment. Plexiform tumors occur in a minority of NF1 patients but are not uncommon [32% by macroscopic examination (Huson et al., 1988); 40% by chest/abdominal/pelvic CT imaging (Tonsgard et al., 1998)]. These tumors are believed to degenerate to more highly transformed malignant peripheral nerve sheath tumors (MPNSTs) in an estimated 6% of cases (Gutmann and Collins, 1995).

NF1 is caused by mutations in the *NF1* gene, a 60-exon tumor suppressor locus at 17q11.2 that encodes neurofibromin (Gutmann and Collins, 1995). Loss of heterozygosity (LOH) for chromosome 17 markers (including the *NF1* locus) has been seen in many NF1 malignancies (Skuse et al.,

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1989; Menon et al., 1990; Glover et al., 1991; Xu et al., 1992; Legius et al., 1993; Lothe et al., 1993, 1995; Shannon et al., 1994; Gutmann et al., 1995; Martinsson et al., 1997; Side et al., 1997). Other studies support a tumor suppressor function, such as neurofibromin's GTPase activating protein (ras-GAP) function, and late-onset tumor predisposition in heterozygous knockout mice (Gutmann and Collins, 1995). Colman et al. (1995) showed LOH involving *NF1* in 8/22 dermal neurofibromas, and Serra et al. (1997) found LOH in 15/60 dermal neurofibromas, with a combined percentage of 28% of these tumors showing *NF1* LOH. The region of *NF1* loss varied in these studies, even among tumors from one patient, supporting the hypothesis that *NF1* loss is an independent, somatic event. Sawada et al. (1996) found a somatic 4 bp *NF1* deletion in a dermal tumor in a patient with a constitutional *NF1* deletion, also supportive of the two-hit hypothesis. The only studies that have examined plexiform tumors used four intragenic *NF1* markers, but no flanking markers, and found LOH in a total of 7/14 independent tumors (Daschner et al., 1997; Kluwe et al., 1999). A study of *NF1* mRNA editing also showed a small degree of conversion to a nonsense codon in dermal and plexiform neurofibromas (with a higher level of editing in malignant NF1 tumors), supporting a minor role for epigenetic *NF1* inactivation in all three types of tumors (Cappione et al., 1997).

Plexiform neurofibromas are the least studied of NF1 tumors, yet understanding the pathogenesis of these tumors is particularly important, because these tumors often cannot be completely removed surgically and seem to have a risk for malignant transformation. For a better study of these, along with a cohort of the other NF1 tumor types for comparison, we analyzed 15 dermal neurofibromas, 10 plexiform neurofibromas, and five MPNSTs for LOH at *NF1* and other chromosome 17 loci, to gather further evidence for the two-hit hypothesis and to evaluate the types and frequencies of somatic chromosome 17 events in these tumors. The status of the *TP53* gene, encoding the important p53 cell cycle control molecule [mutated in half of human cancers (Soussi, 1996)], was also examined.

MATERIALS AND METHODS

Genotyping for LOH

Blood and tumor samples were collected under an approved Institutional Review Board protocol. Patients met diagnostic criteria for NF1 (Gutmann et al., 1997). For tumors showing LOH, the following data are available: 319T was a tumor of unknown location from a patient who was 47 years old; 328T5 was from the back of a 24-year-old; 386T1 was excised from the arm of a 12-year-old; 452T came from the arm of a 19-year-old; 454-v came from the buttock of a 21-year-old; the PD tumor was a congenital orbital plexiform on a patient who had this operation at age 34 years; 396T was a neck MPNST from a 43-year-old; 459T was an MPNST from the leg of a 28-year-old; 441T was an MPNST from the cheek of an 18-year-old. DNA was isolated from blood and tissue samples as described in our previous LOH study (Colman et al., 1995).

To assay for *NF1* LOH, blood samples from patients from whom tumors were available were initially genotyped for the intragenic markers (denoted NF-) listed in Figures 1 and 2 [methods for these polymorphisms are referenced in a table in Colman et al. (1995) and Rasmussen et al. (1998), except for Evi-20 that was done as described in Lazaro et al. (1993)]. The *TP53* intron 1 polymorphism is a pentanucleotide microsatellite that we genotyped with native PAGE and ethidium bromide staining, after digesting the PCR product with *Hpa*II to reduce the polymorphic fragment to the 60–120 bp size range (Futreal et al., 1991). The *TP53* intron 6 polymorphism is an *Msp*I PCR-RFLP (polymerase chain reaction-based restriction fragment length polymorphism) (McDaniel et al., 1991). D17S841, 1863, 1800, 1301, and 784 are microsatellites that were genotyped using standard radioactive PCR under conditions listed in the Genome Database. The 1F10 *Pst*I RFLP was genotyped by DNA PCR using primers we designed [1F10X2: 5′-TTT ACC CTC GGA TAC TGG TGT TGC, and 1F10X3: 5′-GAG TAC CTT GGT GGA GGC CCA CTC] at 65°C annealing, followed by digestion with *Pst*I. The uncut allele is 245 bps in size, and presence of the *Pst*I site cuts the product into fragments of 130 and 115 bps (separated on PAGE, visualized with ethidium bromide). For markers at which the patient was heterozygous, DNA from primary tumor material was also genotyped. For tumors that displayed *NF1* LOH, we tested flanking markers to examine the boundaries of the LOH segment. Genotypes and LOH were confirmed by repeat analysis, in particular the RFLPs, to control for partial enzyme digestion.

NF1 Mutation Detection

The protein truncation test, thought to detect 60–75% of germline mutations, was used to screen

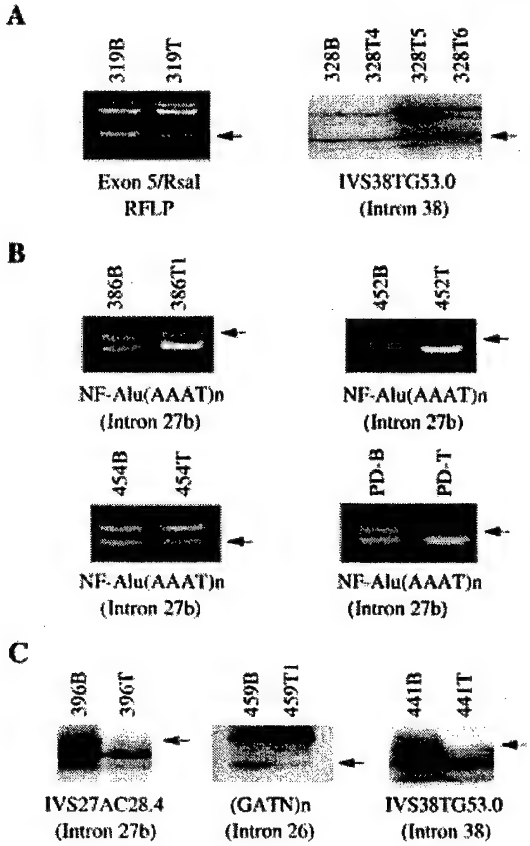


Figure 1. Loss-of-heterozygosity (LOH) analysis. As shown, two dermal neurofibromas (**A**), four plexiform neurofibromas (**B**), and three MPNSTs (**C**) demonstrate *NF1* allele loss (B = blood, T = tumor), whereas two dermal tumors (328T4, 328T6) do not show LOH. The arrow marks the allele that is decreased in intensity, representing LOH.

NF1 mRNA from blood and tumor samples via overlapping RT-PCR fragments, when such material was available (Heim et al., 1995; Park and Pivnick, 1998). Positive PTT signals led to individual examination of the encoded exons by PCR with heteroduplex analysis and SSCP (Abernathy et al., 1997). Individual exons showing electrophoretic abnormalities were directly sequenced using the ABI Big Dye Terminator kit and ABI 377 or 373 automated sequencer, to reveal the mutations.

TP53 Mutation Detection

For all tumors, exons 4–9 were individually amplified (primer sequences and conditions from Oncor, Inc.), and the PCR products were analyzed by heteroduplex analysis and SSCP as above. These six exons are the most commonly mutated exons in *TP53*. PCR products with abnormal electrophoretic patterns were directly sequenced as above. Two positive controls [point substitutions, one in an MPNST (Nigro et al., 1989) and one in an osteosarcoma (data not published)] were used to establish the sensitivity of these assays (particularly SSCP) at detecting even single base changes.

RESULTS

The 15 dermal tumors were obtained from 8 patients. The plexiform tumors and MPNSTs all came from separate patients. Genotyping identified LOH in 2/15 (13%) dermal neurofibromas (2 different patients), 4/10 (40%) plexiform neurofibromas, and 3/5 (60%) MPNSTs, with loss of chromosome 17 markers at least within the *NF1* locus. Figure 1 shows examples of LOH, and an LOH result summary is shown in Figure 2. These results were reproducible, and the level of the residual normal allele was consistent for all loci showing LOH. There is no evidence that any of the LOH regions in dermal or plexiform neurofibromas extend into the p arm (i.e., *TP53* markers). The only MPNSTs that had *TP53* LOH were two of the three MPNSTs that showed *NF1* LOH. *TP53* exon analysis of all tumors failed to reveal any abnormalities, although the two point mutation controls were detected by SSCP, supporting the notion that *TP53* mutations are likely to be absent in exons 4–9 (the "hot spot" exons) in these tumors.

Constitutional mutations were identified in three of the tumors showing LOH, supporting the two-hit hypothesis at *NF1*. In UF452, the germline mutation was found to be C4084T (nonsense mutation R1362X) in exon 23–2 (Fig. 3A), that was maintained in the plexiform tumor sample as assayed by *Taq*I restriction digest analysis (data not shown). In UF459, the germline mutation was found to be a novel single base deletion in exon 21, a frameshift creating a prematurely truncated protein (before the GAP domain) (3683delC). This mutation was also present in the tumor sample (MPNST), indicating that the normal allele was lost in the tumor, fulfilling the two-hit hypothesis (Fig. 3B). UF319's germline mutation is the exon 19b nonsense mutation C3208T (Q1070X at the protein level), and was also retained in the tumor. Germline mutations (some not yet fully characterized) were detected by PTT in several patients whose tumors did not show allelic loss (representing 3 plexiforms, 2 dermal tumors from the same individual, and one MPNST). These aberrant patterns, or the specific mutations, were reproducible in the tumors, also consistent with the two-hit hypothesis (data not shown).

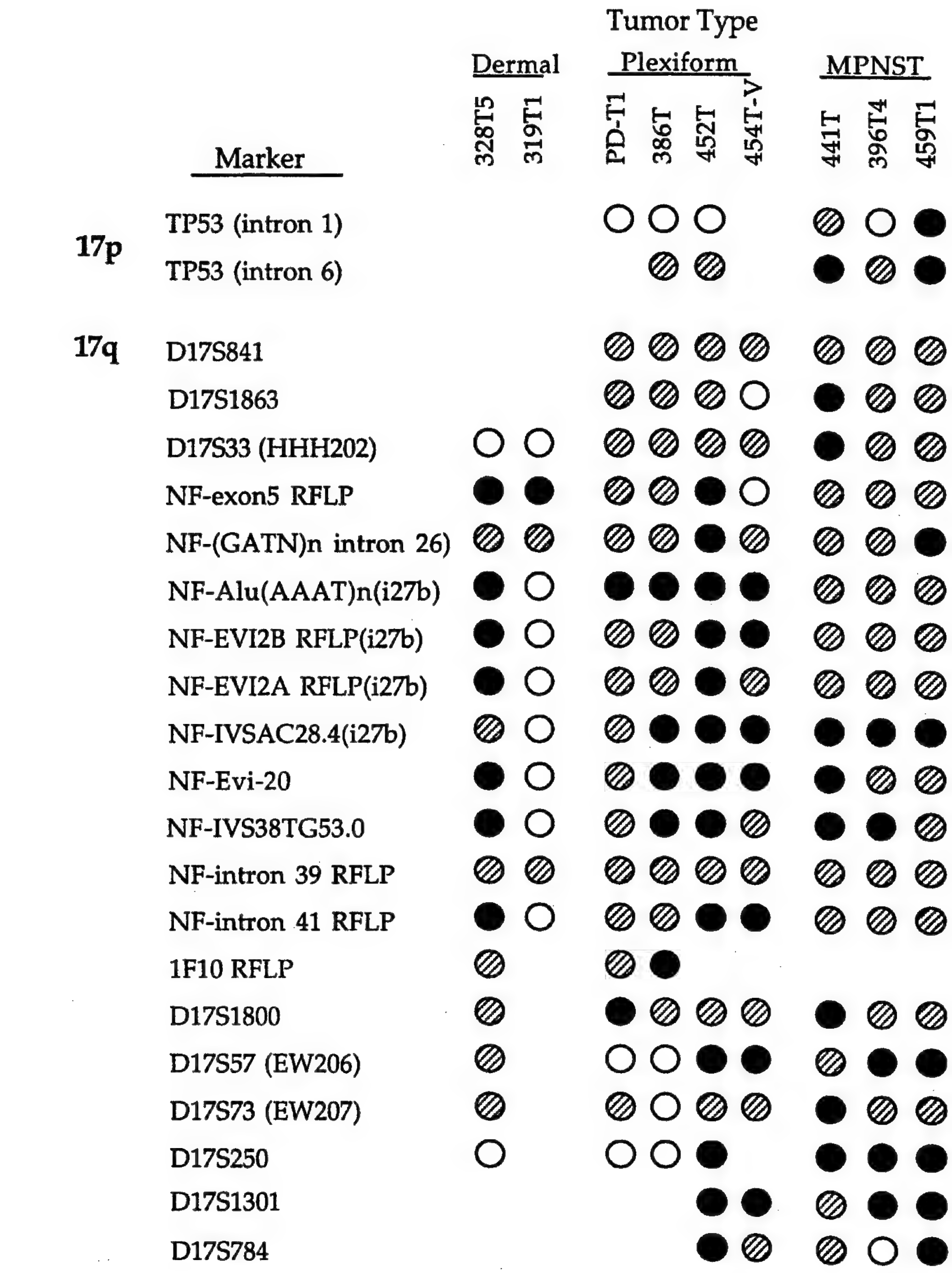


Figure 2. Schematic diagram showing results of loss-of-heterozygosity (LOH) analysis at intragenic *NF1* and flanking markers in the dermal neurofibromas, plexiform neurofibromas, and MPNSTs studied. The markers are listed from pter to qter, in the best estimated order based on published maps. The *TP53* markers are on 17p13.1, and the rest of the markers are on 17q: D17S841–D17S1800 lie in 17q11.2; D17S57, D17S73, and D17S250 lie at 17q12. D17S1301 is not well mapped cytogenetically, but seems to reside in the 17q22–q24 interval based on physical maps. D17S784 is at 17q25 near the telomere. Black circles indicate LOH, striped circles indicate uninformative markers, and open circles indicate heterozygosity (no LOH).

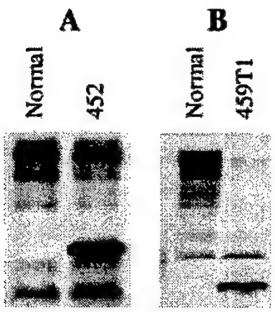


Figure 3. PTT results showing the germline mutations in UF452 (**A**) from leukocytes; and UF459T (**B**) from tumor tissue. The first lane of each panel shows the normal PTT pattern, and the second lane shows the pattern of the patient material.

Parental DNA was analyzed for three patients whose tumors showed LOH (data not shown). For UF328 (a new mutation case), the intron 38 microsatellite showed that the maternal *NF1* allele was lost in the tumor, suggesting that the germline mutation lies on the paternal allele. For PD (also a new mutation case), analysis at D17S1800 (approximately 250 kb downstream of *NF1*) showed that the tumor had lost the maternal allele, with the germline mutation on the paternal allele. Both of these cases are consistent with a preponderance of paternal origin of new *NF1* mutations (Jadayel et al., 1990). In another case, UF319's affected daughter was found to have inherited the allele retained in UF319's dermal tumor, also consistent with the two-hit theory.

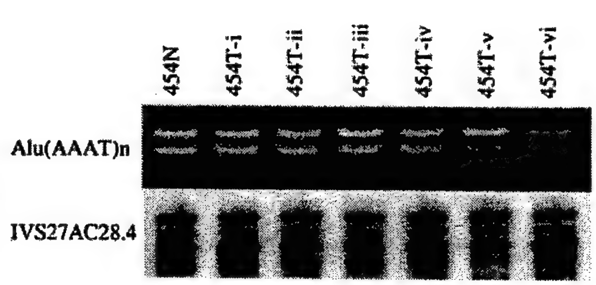
Figure 4 shows results of LOH evaluation of six different portions (small, discrete, firm, individual "worm-like" masses) dissected from within a large diffuse plexiform mass of the upper arm of UF454. Diffuse tumors tend to be thin and spread over a large region, often involving the skin and underlying tissue, in contrast to more typical plexiforms (that are more deeply placed and are somewhat better-defined masses). One of the six portions (T-v, Fig. 4) shows a consistent, although subtle, intensity shift, indicative of LOH at informative markers.

DISCUSSION

Our studies discovered *NF1* loss of heterozygosity in all three types of NF1 tumors: dermal and plexiform neurofibromas, and MPNSTs. The region of loss varied among different tumors and in some cases extended to involve most of the long arm of chromosome 17, similar to previous reports in dermal neurofibromas (Colman et al., 1995; Serra et al., 1997). No allelic loss was detected 5′ of *NF1* in benign tumors. Compared to the dermal neurofibromas (both in our study and in others), a larger proportion of plexiform tumors showed LOH, that usually extended through the q arm (in contrast to the typically more limited LOH extent in our series of dermal tumors, this report and Colman et al., 1995). This is consistent with a postulation that plexiform tumors, being more complex and having greater tumorigenic potential, may harbor more genetic changes than do the simpler dermal neurofibromas (supported by recent cytogenetic data: Wallace et al., 2000). This is also consistent with the theory that dermal neurofibromas may require only a somatic *NF1* mutation, whereas the rarer plexiform tumors develop through additional genetic events. These numbers, however, may reflect a sampling effect, because Serra et al. (1997) found loss of the entire 17q in 7/15 dermal neurofibromas. Thus, it will require additional data to more accurately determine the sizes and frequencies of allelic losses in the different tumor types. The percentage of plexiform neurofibromas with LOH in our study is very consistent with previous reports (Daschner et al., 1997; Kluwe et al., 1999).

One possible explanation for lack of allele loss in some tumors is that a more subtle somatic *NF1* mutation occurred (e.g., a point mutation, small deletion, or insertion) that affected neurofibromin production or function, but would not show LOH. A study of bone marrow from 18 NF1 patients with

Figure 4. Loss-of-heterozygosity (LOH) analysis in different portions of a single diffuse plexiform neurofibroma. Only the fifth portion of the tumor (454-v) demonstrates LOH when compared to normal tissue (454N), with consistent reduced intensity of the lower alleles for intron 27b markers Alu(AAAT)n and IVS27AC28.4.



malignant myeloid disorders, however, did not support this hypothesis (Side et al., 1997): the protein truncation test failed to identify a somatic *NF1* mutation in the nine (out of 18) patients with no tumor LOH. These results were unexpected, given the sensitivity of the PTT, and the fact that small somatic mutations in other tumor suppressor genes are typically truncating (Bijlsma et al., 1994; Polakis, 1995).

In our study, the two MPNSTs that did not show LOH are somewhat suspect in terms of the tissue used for DNA extraction. UF284's tumor was irradiated before surgery, and thus the DNA present in the sample may be from the residual normal cells (if malignant cells had died). UF158's MPNST, although not irradiated before surgery, was noted to have large regions of necrosis, with the most viable tumor only at the margins. Thus, it is possible that there was a minimal contribution of malignant cell DNA to the UF158T sample.

MPNSTs UF441T and UF459T showed LOH at all informative chromosome 17 loci, suggestive of possible homolog loss as previously seen in NF1 MPNSTs (e.g., Glover et al., 1991). UF396T4 showed only 17q loss, however. The lack of *TP53* mutations (in hot-spot exons 4–9) suggests that *TP53* may play a role only in malignant progression of some plexiform tumors [that is similar to its proposed role late in the genetic pathway in colon cancer (Nigro et al., 1989)], or that other *TP53* aberrations are present but were not detected.

Allelic loss on 17q was found in one of six discrete masses dissected from the diffuse plexiform tumor, with the proximal breakpoint in the *NF1* gene. Presumably, LOH is partially masked in this one tumor fragment (UF454T-v), and fully masked in the others due to admixture of normal cells in the sampled tumor segments, because neurofibromas contain a number of different cell types. This "contamination" by normal cells might also explain the lack of detection of LOH in some of the tumors of other patients. Other explanations for the UF454 observation, however, include: (1) several independent genetic events cooperate in a region to produce diffuse plexiform tumors (i.e., the other portions actually do not have LOH), or (2) LOH had occurred in only that portion of the whole tumor, in which case LOH would not be an initiating event for the whole mass.

Further studies will determine critical pathways in NF1 tumorigenesis; it is possible that this may not always involve somatic inactivation of *NF1*. Also, it remains to be seen whether inactivation of *NF1* alone is sufficient for dermal or plexiform tumorigenesis. Identification of genetic events in plexiform tumors may provide clues about etiology, provide therapeutic targets, and determine which tumors are at risk of malignant transformation.

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RESEARCH ARTICLES

Culture of Cytogenetically Abnormal Schwann Cells From Benign and Malignant NF1 Tumors

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Dermal and plexiform neurofibromas are benign peripheral nerve sheath tumors that arise in neurofibromatosis type 1 (NF1). NF1 patients also have an increased risk of malignant peripheral nerve sheath tumors (MPNSTs), thought to arise in a subset of plexiform neurofibromas. Plexiform neurofibroma pathogenesis is poorly understood, despite the serious clinical problem posed by these tumors. The Schwann cell is hypothesized to be the cell type initially mutated and clonally expanded in plexiform neurofibromas. To test this hypothesis and search for genetic alterations involved in tumorigenesis, we established Schwann cell cultures from plexiform and dermal neurofibromas. Cytogenetic abnormalities were identified in 4/6 plexiform cultures (including one from a plexiform with a sarcomatous component) and 0/7 dermal neurofibroma Schwann cell cultures. There were no consistent chromosomal regions involved in the abnormal karyotypes, suggesting that plexiform tumors are heterogeneous and may bear a variety of primary and/or secondary genetic changes. This is the first study to show successful culturing of genetically abnormal Schwann cell lineages from plexiform neurofibromas. Thus, we present the strongest evidence yet to support the theory that the Schwann cell is the central component in the development of plexiform neurofibromas. This is a key finding for NF1 research, which will lead to further studies of the genetic and biochemical pathogenesis of these Schwann cell tumors. *Genes Chromosomes Cancer 27:117–123, 2000.*

INTRODUCTION

Neurofibromatosis type 1 (NF1) is a common autosomal dominant condition characterized by multiple benign neurofibromas, café-au-lait spots, Lisch nodules, and increased risk of malignancy. Dermal neurofibromas (cutaneous or subcutaneous, which usually develop during adolescence and adulthood) are primarily of cosmetic significance. These small tumors, rich in extracellular matrix and sparsely populated by Schwann and mesenchymal cells, involve terminal nerves and have virtually no risk of malignant transformation. In contrast, plexiform neurofibromas are usually congenital, typically involve deep or named nerves, can become very large, and usually cause serious functional impairment. Since plexiform tumors often occur on critical nerves and are not discrete masses, surgical removal is rarely successful, and patients suffer compromised function and may ultimately succumb to their disease. Plexiform tumors develop in 10%–20% of NF1 patients, and these highly cellular masses are believed to progress to highly transformed malignant peripheral nerve sheath tumors (MPNSTs) in an estimated 6% of cases (Gutmann and Collins, 1995).

NF1 is associated with germline *NF1* gene mutations (chromosome band 17q11.2), resulting in absent or abnormal neurofibromin (Gutmann and Collins, 1995). However, NF1 tumor pathogenesis remains poorly understood. Studies have shown *NF1* loss of heterozygosity (LOH) in some benign (Colman et al., 1995; Sawada et al., 1996; Daschner et al., 1997; Serra et al., 1997) and malignant NF1 tumors (Skuse et al., 1989; Menon et al., 1990; Glover et al., 1991; Xu et al., 1992; Legius et al., 1993; Lothe et al., 1993, 1995; Shannon et al., 1994; Martinsson et al., 1997; Side et al., 1997). Thus, cumulative data support the tumor suppressor hy-

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pothesis for NF1 and suggest that at least some neurofibromas contain a clonal cellular element.

Schwann cells are the hypothesized progenitor of neurofibromas, in part because of predominance (40%–80% of cells) and similarities with non-NF1 schwannoma (Peltonen et al., 1988). In addition, neurofibroma Schwann cells have invasive and angiogenic properties, suggesting that these are genetically altered cells (Sheela et al., 1990; Muir, 1995). Although NF1 malignancies have been studied cytogenetically (Riccardi and Elder, 1986; Glover et al., 1991; Jhanwar et al., 1994), there is a lack of such analyses in plexiform neurofibromas, probably due to the heterogeneity of routine primary neurofibroma cultures and the inability of Schwann cells to proliferate in standard culture conditions. To overcome these difficulties and address the issue of tumor cell type, techniques were developed to expand and enrich Schwann cells from NF1 tumors. Cytogenetic studies of these cells subsequently revealed somatic changes in 4/6 plexiform cultures, showing that the tumor cells were successfully cultured and that the Schwann cell is genetically altered in at least some plexiform neurofibromas.

MATERIALS AND METHODS

Culture of Schwann Cells From NF1 Tumors

Resected tumor tissues were washed with Leibowitz L-15 medium containing penicillin and streptomycin. Capsular material was removed and viable tumor isolated. Using cross-scalpels, tumor tissue (1 cm^3) was minced and incubated at 37°C overnight in 10 ml of L-15 medium containing 10% bovine serum, 1.25-U/ml Dispase (Collaborative Research), 300-U/ml collagenase (type XI; Sigma Chemical), and antibiotics. The tissue was dispersed by trituration and strained through a 20-μm mesh nylon screen. The filtrate was diluted with L-15 and centrifuged (500 × g, 5 min). The cell pellet was resuspended in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum and antibiotics, and cells were seeded into tissue culture flasks ($\sim 10^6$ cells/75 cm^2). After 4 days, cultures were detached by trypsinization and passaged 1:4. Two flasks of cells in standard culture were later harvested and stored in liquid nitrogen. Based on a modification of Rutkowski et al. (1995), two flasks were grown on plastic precoated with laminin (10 μg/ml), prepared as described in Muir (1994), in medium containing recombinant human glial growth factor-2 (rhGGF-2; 25 ng/ml). rhGGF-2 treatment caused rapid proliferation of Schwann-like cells that quickly outgrew patches of fibroblastic cells. During subsequent passage, Schwann-like cells were enriched further by differential detachment using mild trypsinization and shaking. The combination of preferential laminin attachment, differential detachment, and selective mitogen treatment with rhGGF-2 yielded highly enriched (> 99%) Schwann-like cell cultures within 2–4 passages (Fig. 1). These cultures were expanded, withdrawn from rhGGF-2 as indicated, and harvested for analyses. rhGGF-2 was generously provided by M. Marchionni (Cambridge Neuroscience).

Immunocytochemical Characterization of Schwann Cell Phenotype

NF1 tumor cultures were examined for immunoreactivity with antibodies to the Schwann cell antigens S-100 (Dako) and the low-affinity nerve growth factor receptor (p75; hybridoma 200–3-G6–4; ATCC). Cultures grown on chamber slides (Nunc) were fixed with 2% paraformaldehyde for 20 min, then washed with PBS containing 0.5% Triton X-100. Nonspecific antibody binding was blocked with PBS containing 0.1% Triton and 10% normal serum (blocking buffer) for 1 hr. Primary antibodies were diluted in blocking buffer and applied to wells for 2 hr at 37°C. Bound antibodies were labeled with peroxidase-conjugated secondary antibodies (1/500, Dako) for 1 hr at 37°C and chromogenic development was accomplished with 3,3′-diaminobenzidine-$(HCl)_4$ (0.05%) and hydrogen peroxide (0.03%) in PBS. Bromodeoxyuridine (BrdU) incorporation in vitro and immunolabeling of BrdU-DNA were performed as described previously (Muir et al., 1990).

Cytogenetic Analysis

Metaphase cells were harvested by standard cytogenetic methods following short (1–3 days) treatment with rhGGF-2. To further increase mitotic activity, cells were trypsinized, allowed to reattach, and harvested within 16 hr. Mitotic arrest was performed by either addition of 50 μl of ethidium bromide (1 mg/ml) and 30-μl Colcemid (10 μg/ml) per 5 ml of culture medium for 1 hr, or by addition of 50-μl ethidium bromide (1 mg/ml) and 40 μl of dilute Colcemid (5 μg/ml) per 5 ml of culture medium for 16 hr (overnight). Cells were released using trypsin to create a cell suspension and exposed to either a 0.075-M KCl hypotonic solution or a one part 0.075-M KCl to one part 0.8% sodium citrate hypotonic solution for 20 min. Cells were subsequently fixed by three washes with

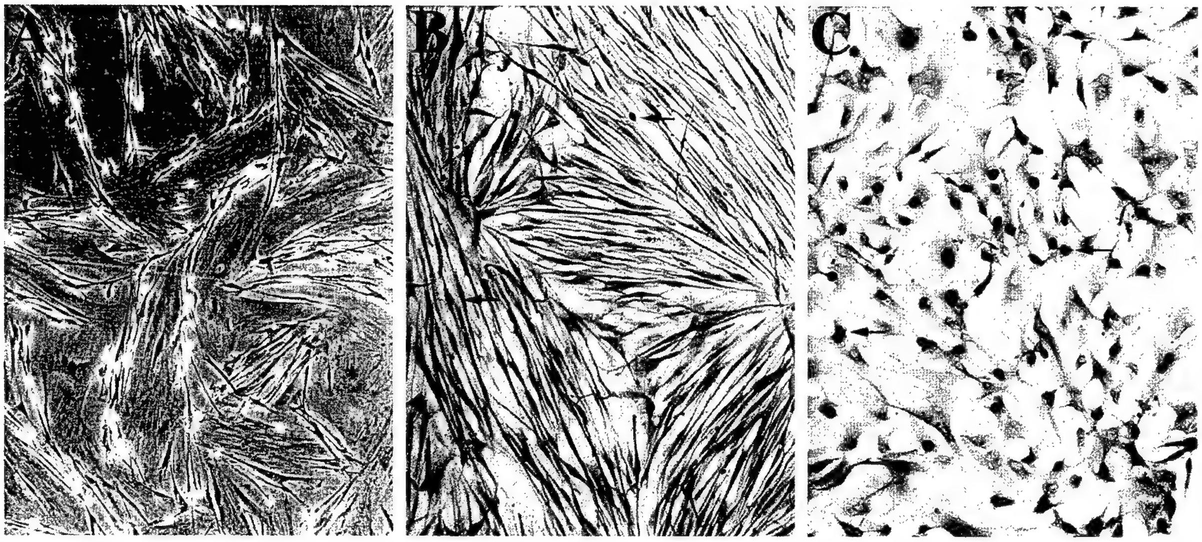

Figure 1. Characterization of SC$^+$ (pNF94.5) and SC$^+$ (pNF92.1) cultures. **A:** Phase contrast photomicrograph of pNF94.5 as a mixed primary culture (passage 1) composed of Schwann cells (phase-bright cell bodies) and fibroblastic cells (asterisk). After Schwann cell enrichment, SC$^+$ (pNF94.5) (passage 3) was withdrawn from rhGGF-2 and grown for 48 hr in medium containing serum and BrdU. **B:** Double immunolabeling for BrdU-DNA (arrows) and S-100 shows SC$^+$ (pNF94.5) mainly contains quiescent Schwann cells (BrdU-negative and S-100–positive cells). **C:** The same double immunolabeling of SC$^+$(pNF92.1) demonstrates that cells of Schwann lineage were highly proliferative in response to serum only after withdrawal of rhGGF-2.

Carnoy's fixative (3:1 methanol:glacial acetic acid). Metaphase spreads were prepared using standard cytogenetic slide making procedures and artificially aged at 54°C for 16 hr. Chromosomes were G-banded (GTG) following modifications of the procedure described by Seabright (1971) and were analyzed by conventional light microscopy. Imaging and karyotyping was performed via standard computer imaging techniques (Applied Imaging).

RESULTS

Primary cultures of the dissociated neurofibromas were composed of spindle-shaped Schwann-like cells and flattened fibroblastic cells. Figure 1A shows plexiform culture SC$^+$ (pNF94.5) in initial stages of Schwann cell enrichment. Schwann cells from neurofibromas generally fail to proliferate under standard culture conditions and are consequently overgrown by fibroblastic cells (Muir, 1995). However, for many neurofibromas, subculture in the presence of the Schwann cell mitogens rhGGF-2 and laminin resulted in highly enriched Schwann cell populations largely devoid of contaminating fibroblastic cells. Only tumor cultures highly enriched in Schwann cells (> 95% S-100–positive cells) were used in this study. Table 1 lists the Schwann cell-enriched (SC$^+$) tumor cultures studied. SC$^+$ cultures from dermal neurofibromas were quiescent or scarcely proliferative in the absence of rhGGF-2. However, this was true for only one-half of the SC$^+$ cultures from plexiform tumors. SC$^+$ (pNF94.5) is an example of a plexiform Schwann cell culture that showed little DNA synthesis in the absence of rhGGF-2 (Fig. 1B). On the other hand, 3/6 plexiform cultures became enriched with a dominant Schwann cell population that continued to expand in serum-containing medium without rhGGF-2. It is important to note, however, that Schwann cell enrichment was vastly improved in the presence of rhGGF-2, a further indication that these cells were of Schwann lineage. BrdU-labeling of one such culture, SC$^+$ (pNF92.1), showed extensive proliferation in the absence of rhGGF-2 (Fig. 1C). SC$^+$ (pNF95.11b) and SC$^+$ (pNF95.6) were the other serum-responsive plexiform Schwann cell lines (see Table 1), but unlike SC$^+$ (pNF92.1), these cultures were derived from classic plexiform tumors with no apparent sarcomatous component. Although different cell morphologies were observed between the different plexiform SC$^+$ cultures, each cell line was mainly homogeneous and immunostained positively for the Schwann cell antigen S-100 (Fig. 1B and C). It is important to note that none of the SC$^+$ cultures were immortalized and all senesced after repeated passage regardless of mitogen stimulation. However, all of the plexiform Schwann cell cultures were expanded beyond the number of passages observed as the limit for Schwann cells derived from normal nerve and the three serum-responsive lines senesced only after protracted expansion (Rutkowski et al., 1995).

TABLE 1. NF1 Neurofibroma Schwann Cell Cultures and Karyotypes

Cell line	Karyotype
Dermal Neurofibromas	
SC+ (cNF96.5f)	46,XX[18]
SC+ (cNF96.5g)	46,XX[13]
SC+ (cNF97.2a)	46,XX[18]
SC+ (cNF97.2b)	46,XX[5]
SC+ (cNF97.5)	46,XY[20]
SC+ (cNF94.5)	46,XY[18]
SC+ (cNF93.1a)	46,XX[10]
Plexiform Neurofibromas	
SC+ (pNF95.6)	46,XY[20]
SC+ (pNF95.11b)	46,XY[20]
SC+ (pNF94.5)	46,XY,t(1;9)(p36.3;p22)[24]
SC+ (pNF95.5)	46,XX,t(2;11)(q13;q23)[3]/
	46,XX,del(12)(q13)[1]/
	91,XXXX,del(2)(p13),idic(4)(q?31.2),−20[1]/
	46,XX[15]
SC+ (pNF95.1)	8/14 cells had unrelated non-clonal abnormalities as described below:
	44,XX,dic(16;20)(p13.3;q13.3)dic(17;22)(p13.3;q13.3)[1]/
	46,XX,+X,−9[1]/
	44,XX,dic(1;17)(q44;p13.3),dic(9;16)(p12;q24),+der(16)t(9;16)(p12;q24), −18,add(22)(q11.2)[1]/
	?46,XX,t(16;22)(p13.3;q13.3)[1]/
	45,XX,del(12)(p13),add(17)(p13.3),dic(22;22)(p11.1;q13.33)[1]/
	45,XX,+1,−12,−16[1]/
	45,XX,−8,add(16)(q24),add(20)(q13.3)[1]/
	45,XX,der(9)(9pter → q34::?::15q26 → 15pter),−10,+12,−15[1]/
	46,XX[6]
SC+ (pNF92.1)[a]	46,XX,t(1;9)(p36.1;q21.2),t(9;12)(p24;q13),t(11;17)(q?14.2;?p13)[2]/
	46,XX,t(2;11)(q21;q13),t(3;19)(p12;q13.1),del(6)(q21.2),add(10)(p15), del(14)(q24),add(22)(p11.1)[2]/
	53 ~ 58,XX + 2,add(2)(p21)×2,add(2)(q37),+6,+7,add(7)(q34),+8, add(11)(p11.2),add(11)(q23), −13,add(14)(p11.1),+16,add(16)(q12.1),+17,+19, add(19)(q13.3),+20,+21,−22[cp3]/
	100 ~ 116,XXXX,−X,idemx?[cp2]/[b]
	44,XX,−6,−17,−18,+21[1]/
	46,XX,add(22)(q13)[1]/
	47,XX,+5[1]/
	46,XX[4]

[a]This plexiform tumor had malignant foci.
[b]These two near-pentaploidy cells included the same structural abnormalities observed in the composite karyotype described immediately above; however, the exact copy number could not be established.

As shown in Table 1, no cytogenetic abnormalities were found in the seven cultures derived from dermal neurofibromas. However, 4/6 plexiform tumor cultures showed karyotypic aberrations. In SC$^+$ (pNF94.5), all metaphases contained an apparently balanced translocation. The karyotype was 46,XY, t(1;9)(p36.3;p22) (Fig. 2). A culture from a dermal neurofibroma from the same patient showed only a normal karyotype (18/18 cells, data not shown), demonstrating that the translocation was not constitutional. To test whether the chromosome abnormality in SC$^+$ (pNF94.5) might have been an artifact (selective subculture during Schwann cell enrichment), a second, independent culture of pNF94.5 was also studied. This culture, derived from a separate vial of cells frozen from the first passage, contained a mixture of predominantly Schwann cells and fibroblasts (as in Fig. 1A). The culture was stimulated with rhGGF-2 to induce Schwann cell mitosis and then karyotyped. The t(1;9) was evident in 14/14 metaphases, showing that the translocation is a somatic mutation in the primary tumor and was present prior to Schwann cell enrichment. Two cultures, SC$^+$ (pNF95.6) and SC$^+$ (pNF95.11b), showed no cytogenetic rearrangements. However, these cultures have several tumorigenic characteristics in addition to rhGGF-2–independent growth (data not shown), suggesting that underlying genetic abnormalities likely exist that are not detectable at the cytogenetic level.

SC$^+$ (pNF92.1), a plexiform neurofibroma with sarcomatous foci, showed a complex karyotype with three abnormal clones (see Table 1). Two of 16 cells showed three translocations [t(1;9),t(9;12),t(11;17)].

Figure 2. Karyotype of SC+ (pNF94.5) Schwann-enriched culture, showing the t(1;9)(p36.3;p22) (breakpoints indicated by arrows) at a 400-band level.

A second unrelated clone (two cells) was more complex, with translocations, deletions, and unbalanced translocations, although none were the same as in the previous clone. Three cells contained varying abnormalities with 53–58 chromosomes, including multiple marker chromosomes and material of unknown origin attached to several chromosome arms. The remaining five abnormal metaphase cells contained various aberrations ranging from a simple trisomy 5 to near-pentaploidy; the two near-pentaploidy cells also contained some of the same structural abnormalities seen in the three cells above. It should be noted that this tumor was irradiated after its first resection years earlier; our specimen was this tumor's recurrence and it is unclear whether the irradiation therapy may have affected the karyotype or contributed to the development of the malignancy. All other tumors studied here were devoid of malignant foci and the patients had not received radiation or chemotherapy.

Plexiform SC+ (pNF95.5) had aberrations in 5/20 cells (Table 1). Three of these cells had a t(2;11)(q13;q23); one was 46,XX,del(12)(q13), and the other cell was nearly tetraploid with a deletion of 2p and an isodicentric chromosome 4. The other abnormal culture SC+ (pNF95.1) had cytogenetic aberrations in 8/14 metaphases, with each abnormal cell being unique. However, all of the structural abnormalities appeared to involve telomeres. In particular, 9q, 16q, 17q, and 20q were involved; some rearrangements resulted in dicentric chromosomes by end-to-end fusions (telomere associations).

DISCUSSION

The four cases with cytogenetic abnormalities are the first somatic mutations discovered in Schwann cells cultured from plexiform neurofibromas, and the significance is twofold. First, this shows that our culture conditions favor tumor cell growth and enrichment (to homogeneity in at least one case) and strongly implicates the Schwann cell as an abnormal element in neurofibromas that is somatically mutated and clonally expanded. This accomplishment vastly improved our ability to

examine the cytogenetic pattern of this cellular component of initially heterogeneous NF1 tumors. This led to the second significant finding, namely, cytogenetic abnormalities, which suggests that chromosome abnormalities appear to be a factor in plexiform neurofibroma development.

Tumor cytogenetic analysis is often useful in identifying gene regions involved in tumorigenesis. Previous studies of malignant NF1 tumors have revealed complex karyotypes (Riccardi and Elder, 1986; Decker et al., 1990; Glover et al., 1991; Rey et al., 1993; Jhanwar et al., 1994; Mertens et al., 1995; Rao et al., 1996), as seen in SC^+ (pNF92.1). SC^+ (pNF92.1) did not contain the same rearrangements in either of the two clones or in the nonclonal abnormal cells, suggesting that genetic instability might be involved in this tumor. The possibility of genetic instability leading to the aberrations must also be considered, in particular for SC^+ (pNF95.1), which shows telomere breaks, since telomere associations are most frequently reported in chromosome breakage syndromes and tumors (Sawyer et al., 1996). This suggests that mechanisms such as loss of integrity of the DNA repair system might be involved in a proportion of plexiform neurofibromas.

Dermal neurofibromas have been previously karyotyped (as primary tissue), with predominantly negative results (Glover et al., 1991), although one was reported with complex karyotypes in 8/33 cells (Riccardi and Elder, 1986). Our current study found no cytogenetic abnormalities in seven dermal neurofibroma Schwann cell cultures. Combined with the literature, this suggests that either the Schwann cell is not the tumor clonal cell type, or the culturing did not successfully enrich for neoplastic cells, or chromosomal abnormalities are infrequent in the tumor Schwann cell population. Given these tumors' slow and limited growth, and lack of malignant potential compared to plexiforms, the last possibility seems most likely.

Our results support the notion that genetic loci other than *NF1* may be of importance in formation of plexiform neurofibromas and MPNSTs. Since rearrangements were not consistent among the abnormal cases, the idea of a single common pathway in NF1 tumorigenesis is not supported. While there is no direct evidence that the abnormalities are causally related to the tumor development, it is possible that genes in the breakpoint or aneuploid regions (particularly in abnormal clones) contribute to the phenotype. For example, one could speculate that the pNF94.5 1;9 translocation affects the *CDKN2A* gene, which lies at 9p21–22 and is involved in multiple tumor types (Kamb et al., 1994; Martignetti et al., 1999). Another observation suggesting possible involvement of this region in NF1 is a constitutional 9p21 deletion found in a non-NF1 patient with a plexiform tumor (Petty et al., 1993). Likewise, the pNF94.5 1p36 breakpoint might affect the *p73* gene (Jost et al., 1997), which encodes a protein with function similar to p53, which in turn has been implicated in some NF1 malignancies (Nigro et al., 1989; Menon et al., 1990; Glover et al. 1991, Xu et al., 1992; Legius et al., 1994; Lothe et al., 1995).

The data presented here support the long-held hypothesis that plexiform neurofibromas harbor clonally abnormal Schwann cells, a key finding for the NF1 research arena. The Schwann cell subculture method is a valuable tool to provide enriched and expandable tumor material in a manipulatable system for future molecular and cellular experiments. Progress in understanding the genetic and biochemical pathways in benign and malignant NF1 tumor formation will be hastened by the use of these cultures, and the data and cultures will be crucial in developing targets for and testing therapeutic interventions.

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Exon 10b of the *NF1* gene represents a mutational hotspot and harbors a recurrent missense mutation Y489C associated with aberrant splicing

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Purpose: To analyze the spectrum and frequency of *NF1* mutations in exon 10b. **Methods:** Mutation and sequence analysis was performed at the DNA and cDNA level. **Results:** We identified nine exon 10b mutations in 232 unrelated patients. Some mutations were recurrent (Y489C and L508P), others were unique (1465–1466insC and IVS10b+2delTAAG). Surprisingly, at the RNA level, Y489C causes skipping of the last 62 nucleotides of exon 10b. Another recurrent mutation, L508P, is undetectable by the Protein Truncation Test. **Conclusion:** As exon 10b shows the highest mutation rate yet found in any of the 60 *NF1* exons, it should be implemented with priority in mutation analysis. ***Genetics in Medicine*, 1999:1(6):248–253.**

Key Words: *Neurofibromatosis type 1, mutation, hotspot, aberrant splicing, polymorphism, MIR*

Neurofibromatosis type 1 (NF1, MIM 16,2200) is one of the most common autosomal dominant disorders, affecting about 1:3500 individuals in all ethnic groups. The main characteristics are cutaneous or subcutaneous neurofibromas, café-au-lait (CAL) skin spots, iris Lisch nodules, and freckling.[1] Other features found in only a minority of patients include scoliosis, macrocephaly, pseudarthrosis, short stature, malignancies, and learning disabilities. As NF1 clinically presents with great variability, even among patients in the same family, the pathogenicity underlying the phenotype must be complex.

The *NF1* gene has been mapped to 17q11.2 and was positionally cloned.[2–4] The *NF1* gene is approximately 350 kb in size, contains 60 exons and codes for a 11- to 13-kb transcript with an open reading frame coding for 2818 amino acids.[5] By RT-PCR, *NF1* mRNA appears to be ubiquitous, but by Western blot, neurofibromin is best detected in neural-crest-derived tissues.

The mutation rate in the *NF1* gene is one of the highest known in humans (reviewed by Huson and Hughes[1]) with approximately 50% of all NF1 patients presenting with new mutations. Despite the high frequency of this disorder in all populations, relatively few mutations have been identified at the molecular level, with most unique to one family. A limited number of mutational "hotspots" have been identified: R1947X (C5839T) in exon 31 and the 4-bp region between nucleotides 6789 and 6792 in exon 37, both implicated in about 2% of the NF1 patients (reviewed by Upadhyaya and Cooper[6]). In this study we report that another mutational hotspot resides in exon 10b. By analyzing altogether 232 unrelated NF1 patients, we identified 9 mutations in exon 10b, indicating that this exon is mutated in almost 4% of NF1 patients. So far this is the highest score obtained for a particular exon in the 9 years since the *NF1* gene was discovered.

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MATERIALS AND METHODS

NF1 patients

For all patients, the diagnosis of NF1 was based on the presence of two or more of the diagnostic criteria proposed by the NIH Consensus Statement in 1988[7] and updated in 1997.[8] The study was approved by the Institutional Ethical Committees and informed consent was obtained from the patients studied. Patients were recruited randomly without bias as they were seen for medical follow up and genetic advice. Patients were recruited as part of a general mutation study. Seventy-three patients were contributed by the Department of Medical Genetics of Gent and by the Service de Genetique, Hopital Erasme, Brussels; 159 patients were contributed by the Department of Pediatrics, Division of Genetics, University of Florida, Gainesville, and by the Departments of Neurology and Medicine, Center for Human Genetics, Duke University Medical Center, Durham.

Nucleic acid extraction

DNA and RNA samples were obtained from 37 unrelated NF1 patients by extraction from EBV-transformed lymphoblastoid cell lines. In addition, DNA and total RNA samples

were obtained from 195 unrelated NF1 patients by extraction from peripheral blood lymphocytes. Total cellular RNA and genomic DNA was isolated as described.[9]

cDNA analysis and in vitro transcription/translation analysis

First strand cDNA was synthesized by random priming[9] and cDNA was amplified using 5 primer pairs for amplification of the total coding region.[10] 4 μL PCR product was used in an optimized in vitro transcription/translation reaction as described.[9,11] An identical truncated peptide fragment of 55 kD was observed in 2 of 37 patients by in vitro transcription/translation of the fragment spanning exons 1 to 12a and the corresponding cDNA was analyzed by cycle sequencing with and without subcloning using 0.15 μM fluorescein isothiocyanate (FITC) labeled primers, designated by nucleotide positions: 5′-CTTCGGAATTCTGCCTCT-3′ (400–418), 5′-CTGATATGGCTGAATGTG-3′ (719–736), 5′-GCCTGTGTCAAACTGTGT-3′ (967–984), and 5′-CACACCCAGCAATACGAA-3′ (1367–1384), and the Thermo Sequenase fluorescent labeled primer cycle sequencing kit (Amersham, Arlington Heights, IL). Samples were loaded on a 6% LongRanger gel (FMC, Rockland, ME) containing 7 M urea and analyzed on an ALF automated DNA sequencer. To check for the presence of the missense mutation Y489C in a fraction of the cDNA, RT-PCR fragments were cloned using the pCR-TOPO cloning kit (InVitrogen, San Diego, CA) and 90 individual clones were further analyzed by cycle sequencing.

Genomic DNA analysis

Exon 10b was amplified using the primer pair as described[12] and PCR products were further analyzed by cycle sequencing without subcloning.[9] Genomic DNA from 195 unrelated NF1 patients from which no EBV lymphoblastoid cell line was available was analyzed by heteroduplex analysis or single strand conformation analysis or both, and aberrant migrating PCR fragments were further analyzed by cycle sequencing.[13] Mutations are reported according to the recommendations of the Nomenclature Working Group,[14] with the start site of translation denoted as nucleotide 1 for both cDNA and genomic alterations.

RESULTS

The total coding region of the *NF1* gene was analyzed by the protein truncation test in 37 unrelated NF1 patients from which an EBV lymphoblastoid cell line was available.[10] In two patients, an identical shortened fragment of approximately 55 kD was discerned in the region encompassing the exons 1 to 12a. In both patients, in vitro transcription/translation for the other regions only showed normal sized fragments. By electrophoresis of the RT-PCR fragments from Patient 1 two discrete bands were discerned on a 1.5% agarose gel, i.e., a normal-sized band of 1868-bp and a band that was approximately 60 bp smaller. In Patient 2, however, only a normal-sized band was seen, indicating that the truncated protein of identical size was formed in a different way in this patient. cDNA sequencing in this region indeed revealed a different mutation in both patients. In Patient 2, an insertion of C at nt 1465–1466 in exon 10b was found, immediately resulting in the creation of a stop codon at this site. In Patient 1, a deletion/skipping of the last 62 nucleotides of exon 10b was observed in RNA from both lymphocytes and the EBV-lymphoblastoid cells (Fig. 1B). Here too, the immediate result is formation of a stop codon at this site, explaining the identical picture seen by protein truncation analysis. Further analysis of exon 10b at the genomic level confirmed the presence of an insertion 1465insC in Patient 2. In Patient 1, however, a missense mutation was identified: A1466G, changing the codon for Tyr to Cys (Y489C) (Fig. 1A). Both parents of this sporadic patient did not carry this missense mutation. This missense mutation masquerades as a splicing defect: indeed substitution of A to G at position 1466 of the genomic DNA creates a new splice donor site (CT/GTAAG) (Fig. 1C). Analysis of the normal and mutant se-

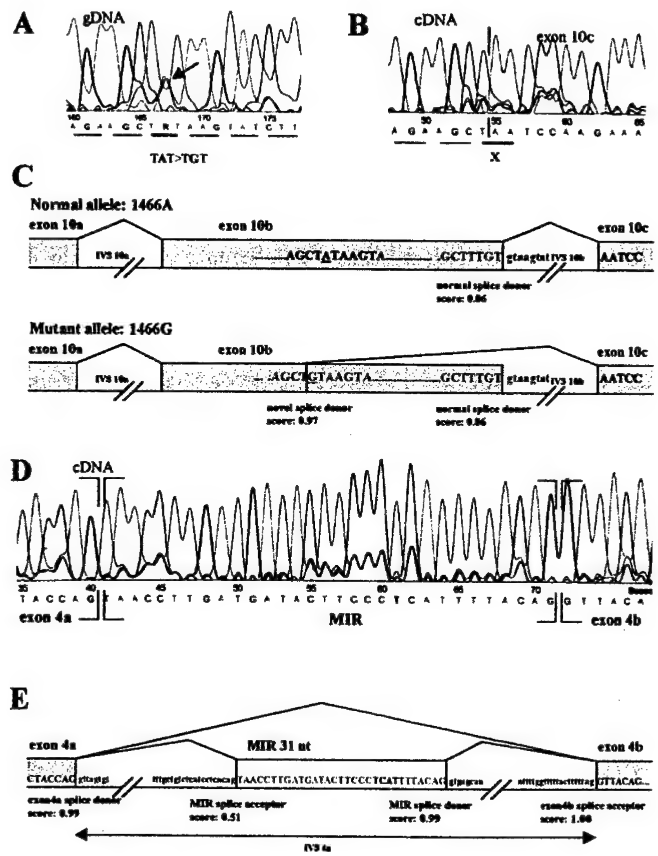

Fig. 1 (A) Cycle sequencing without subcloning of the genomic region of exon 10b in Patient 1 showing transition of A to G at nt 1466 resulting in the formation of a splice donor site CT/gtaag; (B) Cycle sequencing of the mutant cloned cDNA allele in Patient 1. The last 62-bp of exon 10b are skipped and followed by exon 10c, resulting in the formation of a stop codon at amino acid 489; (C) Schematic diagram of the genomic region surrounding exon 10b. Green boxes represent exons, red sequences denote splice donor sequences. By the transition of A to G at nt 1466 a novel splice donor is formed; (D) Cycle sequencing of a cloned RT-PCR fragment encompassing the exons 1 to 12b using sequencing primer 5′-CTTCGGAATTCTGCCTCT-3′ (400–418). Between exon 4a and 4b the presence of the 31-bp MIR-sequence is shown; (E) Schematic diagram of the genomic region surrounding IVS 4a. Green boxes represent exons, red sequences denote splice donor sequences, green sequences denote splice aceptor sequences.

quence using the program for splice site prediction by neural network (available at URL: www-hgc.lbl.gov/projects/splice.html) showed a 0.86 score for the normal exon 10b donor site (GCTTTGT/gtaagtat) and a higher 0.97 score for the new donor site created by the missense mutation Y489C (AGAAGCT/gtaagtat). RT-PCR fragments from an EBV lymphoblastoid cell line of Patient 1 were cloned and 90 individual clones were further analyzed by cycle sequencing to check for the presence of the missense mutation in a fraction of the cDNA. In 50 cDNA clones showing a normal-sized band of 1868 bp on a 1.5% agarose gel, only the wild type sequence was found and in none of them the missense mutation was present. In 40 clones containing a slightly smaller insert (approximately 60 bp) as evidenced by agarose gel electrophoresis, the smaller size was due to the skipping of the last 62 nucleotides of exon 10b along with intron 10b in the mRNA (Fig. 1B). This indicates that the major outcome of the mutation Y489C at the cDNA level is skipping of the last 62 nucleotides of exon 10b. Although Y489C and 1465–1466insC are different mutations at the genomic DNA level, both result in the formation of a premature stop codon at exactly the same position well before the GAP domain of neurofibromin. As the finding of two different mutations at the same spot (i.e., 1465–1466insC and 1466A>G) is in itself indicative of a mutational hotspot,[15] these findings prompted us to analyze exon 10b in a larger patient population consisting of 195 unrelated NF1 patients from which genomic DNA and total RNA was available but no EBV lymphoblastoid cell line. In Patients 3, 4, 5, and 6 the missense mutation Y489C was identified by heteroduplex analysis or single strand conformation analysis or both, followed by cycle sequencing of the aberrant migrating genomic fragments and further analysis at the cDNA level. In Patients 4, 5, and 6, the effect of the missense mutation Y489C at the mRNA level was identical to Patient 1, i.e., skipping of the last 62 nucleotides of exon 10b. Surprisingly, three different sized transcripts were seen after RT-PCR of total RNA extracted from fresh lymphocytes from Patient 3 (no EBV lymphoblastoid cell line was available from this patient). RT-PCR fragments were cloned and further analyzed by cycle sequencing. In the smallest sized fragments, the mutant exon 10b lacking the last 62 bp of the exon 10b was followed by the correct exon 10c but hereafter the exon 11 was neatly skipped out. Skipping of exon 11 was not seen in Patients 1, 4, 5, and 6. In the clones carrying the intermediate-sized transcripts, the mutant exon 10b lacking the last 62-bp was found. In the largest fragments, the normal exon 10b sequence was present as expected. Furthermore, between exon 4a and exon 4b, a 31-bp cryptic exon was inserted in *cis* with the skipping of the last 62 bp of exon 10b and exon 11 in some of the mutant clones (Fig. 1D). This 31-bp cryptic exon was also seen in some mutant clones lacking only the last 62 bp of exon 10b, but also in some normal clones. The same cryptic exon was recently found in a patient showing multiple splicing errors but an unknown constitutional mutation.[16]

In Patient 7, a deletion at the splice donor site of exon 10b was found (IVS10b+2delTAAG) and analysis of RNA extracted from a neurofibroma showed a strong shorter RT-PCR product in addition to the normal sized fragment. By cycle sequencing, it was shown that the shorter transcript was formed by the perfect splicing out of exon 10b. As this splicing alteration resulted in the production of a stable mRNA and the size of exon 10b is a multiple of three, the possibility remains that this allele produces neurofibromin lacking only the 45 amino acids encoded by exon 10b.

In two unrelated NF1 patients (8 and 9; one Caucasian and one African American) an identical missense mutation was identified changing the codon for Leu to Pro (L508P; 1523T>C). This missense mutation that would not be detected by the Protein Truncation Test, does not create/destroy a restriction enzyme site, but shows a specific reproducible shift in single strand conformation polymorphism analysis. Analysis of 153 unrelated Caucasian Americans and 20 unrelated Black Americans ruled out that the alteration is a polymorphism. Another 30 unrelated Caucasian Europeans were analyzed by direct sequencing of exon 10b. In none was the missense mutation L508P found. Table 1 summarizes the effects on RNA splicing of the different germline mutations found in exon 10b.

A novel polymorphism was identified at a CpG dinucleotide in intron 10a: IVS10a-32C>T. We have tested 61 unrelated NF1 patients and 72 normal control persons for this polymorphism and no statistically different values were obtained between the two populations. Taking both populations together, we obtained allele frequencies of 0.68 for the IVS10a-32C allele and 0.32 for the IVS10a-32T allele. In four NF1 patients heterozygous for this polymorphism, we analyzed by RT-PCR the outcome of the IVS10a-32T allele with respect to the splicing of exon 10b and did not observe an effect on the splicing. As the number of polymorphisms at the 5′ end of the *NF1* gene is limited, this novel polymorphism, which can be detected by *Mae*II digestion, may be a useful tool in segregation analyses.

DISCUSSION

In this study we identified nine novel exon 10b mutations in 232 unrelated NF1 patients: Y489C or 1466A>G (5X), L508P (2X), 1465–1466insC (1X), and IVS10b+2delTAAG (1X). Our findings indicate that exon 10b is mutated in almost 4% of the NF1 patients, although so far, only one missense mutation has been reported in exon 10b, K505E.[16] However, only two studies analyzed the complete coding region of the NF1 gene including exon 10b, and the total number of patients analyzed in both studies is small, i.e., 36 patients.[10,16] No other studies specifically looked at exon 10b. Mutational analysis is hampered by the large number of exons, the wide variety of mutations, and the presence of several pseudogenes. The majority of mutations identified hitherto reside in the GAP-related domain, encoded by the exons 21 to 27a and in the exons downstream of this GAP-related domain. The exons lying at the 5′ end of the gene have not been studied exhaustively by many groups. One reason may be that for most exons at the 5′ end the available intronic sequence information was limited to the

Table 1
Summary of the germline mutations found in exon 10b and their effects on RNA splicing

Patient	gDNA mutation	Tissue(s) analyzed ● cDNA observations
P1	Y489C; 1466A>G	EBV lymphoblastoid cell line and fresh lymphocytes
		● skipping of 62 nt at cDNA nt 1466–1527 resulting in formation of a stop codon at AA 489
P2	1465–1466insC	EBV lymphoblastoid cell line
		● formation of a stop codon at AA 489
P3	Y489C; 1466A>G	Fresh lymphocytes
		● skipping of 62 nt at cDNA nt 1466–1527 resulting in formation of a stop codon at AA 489
		● skipping of 62 nt at cDNA nt 1466–1527 AND skipping of exon 11
		● insertion of a cryptic 31 nt exon, between exon 4a and exon 4b resulting in formation of a stop codon at nt 516 of the cDNA, both on the normal and mutant allele
P4	Y489C; 1466A>G	Fresh lymphocytes
		● skipping of 62 nt at cDNA nt 1466–1527 resulting in formation of a stop codon at AA 489
P5	Y489C; 1466A>G	Fresh lymphocytes
		● skipping of 62 nt at cDNA nt 1466–1527 resulting in formation of a stop codon at AA 489
P6	Y489C; 1466A>G	Fresh lymphocytes
		● skipping of 62 nt at cDNA nt 1466–1527 resulting in formation of a stop codon at AA 489
P7	IVS10b+2delTAAG	RNA from a plexiform neurofibroma (blood RNA not available)
		● skipping of exon 10b
P8	L508P (1523T>C)	No RNA available
P9	L508P (1523T>C)	Fresh lymphocytes
		● no effect on splicing of exon 10b

intron-exon boundaries, thus hampering the search for optimal primer pairs that can be used to amplify these exons, in the absence of sequences derived from pseudogenes.

The novel mutations 1465insC and Y489C are different at the genomic DNA level, but both result in the formation of a premature stop codon at the mRNA level at exactly the same position through different mechanisms. Y489C illustrates that, although the mutation is reported according to the recommendations of the Nomenclature Working Group, nomenclature can be misleading. Indeed, by nature Y489C belongs to the group of splicing error mutations and not to the group of missense mutations. This unexpected finding further stresses the importance of documenting the outcome of a mutation at the mRNA level.

It is intriguing that in 1 of the 5 patients carrying the mutation Y489C, analysis of cloned RT-PCR products showed that some mutant alleles skipped the last 62 bp of exon 10b as well as the complete exon 11. Exon 10c was retained in these clones. Skipping of exon 11 was not seen in the other patients. The cause of the exon 11 skipping in Patient 3 is unknown and may not even be due to a sequence change in the *NF1* gene, or it could be caused by a point change in an intron that renders this exon skipping more likely to occur. As we do not have an EBV lymphoblastoid cell line from this patient, we were not able to study whether this different splicing is tissue-specific. Noteworthy is that, by splice site prediction using neural networks (available at: URL: www-hgc.lbl.gov/projects/splice.html), no splice acceptor sequence can be defined for exon 11, not even after using an acceptor score cutoff of 0.05. This might indicate that splicing of exon 11 is mediated through mechanisms different from splicing of those exons with a well-defined splice acceptor sequence.

The cryptic 31-bp exon between exon 4a and exon 4b was found on normal as well as on mutant cloned cDNA fragments of Patient 3. From ongoing experiments we know that this cryptic 31-bp exon is formed, albeit typically at a low level, in different cell types in NF1 patients as well as in normal persons, and hence does not appear associated with a mutation in the *NF1* gene per se.[17] In some neurofibromas, the cryptic 31-bp exon can be found on both alleles as well (unpublished results). A search of sequence databases showed that this 31-bp sequence was 100% homologous with a part of the *NF1* intron 4a. Using the splice site prediction by neural network program we found that this sequence is flanked by a splice acceptor sequence (score 0.51) tttgctgtctcatcctcacag/TAACCTTGATGATACTTCCC and a very strong splice donor sequence (score 0.99) TTTACAG/gtgagcaa (Fig. 1E). The mechanisms by which this splice acceptor/donor pair is up- or downregulated is unknown, nor is it known whether this transcript is translated in vivo. The 31-bp cryptic exon is part of a fragment of the ancient class of Mammalian-wide Interspersed Repeat

Table 2
Summary of the clinical findings in the patients carrying a mutation in exon 10b of the NF1 gene

Trait	P1	P2	P3	P4	P5	P6	P7	P8	P9
Sex	F	F	M	M	F	F	F	M	M
Age (Yr)	3	7.5	39	9	48	23	18	80	17
Ethnicity	Caucasian	Caucasian	Caucasian	Caucasian	Caucasian	Caucasian	Caucasian	Caucasian	Black American
CALs	>6 and >5mm	>6 and >5mm	>6 and >15mm	>6 and >5mm	>6 and >15mm	>6 and >5mm	>6 and >15mm	>6 and >15mm	>6 and >15mm
Cutaneous NFs	No	Yes (subcutaneous)	Yes	No	Yes	Yes	Yes	Yes	Yes
Plexiform NF	Yes: neck and left hemithorax	Yes: paravertebral	No	No	No	Several larger NFs that may be plexiforms	Yes: of left hand	No	Yes: periorbital
Freckling	Yes	ND	Yes	Yes	ND	Yes	Yes	Yes	Yes
Optic glioma*	No	ND	No	No	No	No	No	ND	ND
Lisch nodules	No	ND	Yes	Yes	Yes	No (no slit lamp exam)	Yes	ND	Yes
Osseous lesion	Hypoplasia of the left thoracic pedicles from thoracic vertebrae I–VII; thoracic scoliosis	Scoliosis requiring osteosynthesis	No	No	Scoliosis; no bone dysplasia	Scoliosis	No	No	No
First-degree relative with NF1	No	Yes	Yes	No	No	No	No	ND	No
Other manifestations	Macrocephaly	Macrocephaly	Complex partial epilepsy; mentally functioning at a lower level	Macrocephaly, learning disabilities	Seizures	Xanthogranulomas	Short stature, learning disabilities	None	Learning disabilities; macrocephaly

M = male, F = female.
*Presence/absence of optic pathway glioma as evidenced after MRI evaluation; ND, not determined.

elements (MIR) (as defined in Genbank sequence AC004222, nt 22263 to 22346 and confirmed by A.F.A. Smit and P. Green, [personal communication, The Genome Center, Washington, DC, 1998] using the program RepeatMasker [Available at: http://ftp.genome.washington.edu/RM/RepeatMasker.html]). Further research into the direct outcome of this germline mutation at the mRNA level in different tissues and cell types in the different affected persons from this family, and comparison with the nonaffected family members as a control may shed more light on this event.

The missense mutation L508P was identified in two unrelated NF1 patients and absent in 203 unrelated control persons from different ethnic origin. Proline is a very rigid amino acid and its presence creates a fixed kink in a polypeptide chain. Although both amino acids are hydrophobic and the amino acid change is conservative, the introduction of a nonaliphatic imino group into the polypeptide may have dramatic consequences for protein structure. Moreover, leucine at this position is conserved in the mouse (L10370), rat (D45201), Fugu rubripes (AF064564), and drosophila (L26501). We presume that this missense mutation is pathogenic and may point to a region of the protein with a hitherto unknown function.

NF1 genotype-phenotype correlation studies have not been extensive or successful except for the consistent correlation between the deletion of the entire *NF1* gene and flanking region and a distinctive phenotype including severe developmental impairment, dysmorphic features, and large numbers of neurofibromas.[18–20] Detailed clinical data together with the genotypes of the patients were submitted to the NNFF International *NF1* genetic mutation database, to allow NF1 genotype-phenotype correlation studies in the future. In 4 of 9 patients scoliosis was present (Table 2). It is worth noting that in our patients with a mutation in exon 10b, a high proportion (5 of 9) harbored plexiform neurofibromas (Table 2). Patient 3, who does not have plexiform neurofibromas himself, has a child with a plexiform neurofibroma on the lip.

In conclusion, a novel mutational hotspot has been identified in exon 10b of the *NF1* gene: a mutation in exon 10b was found to be present in 9 of 232 (3.9%) unrelated NF1 patients. By cDNA-SSCP analysis of the total coding region in 71 Spanish NF1 patients, an identical skipping of nt 1466–1527 of exon 10b caused by the mutation Y489C was found in two patients (E. Ars and C. Lazaro, personal communication, Medical and Molecular Genetics Center–IRO, Barcelona, Spain). The finding of another missense mutation in exon 10b (K505E) in 1 of 14 unrelated American patients[16] further underscores this finding. Taking these data together, 12 exon 10b mutations were reported in 317 unrelated NF1 patients (~3.8%) and is, thus far, the highest score obtained for an *NF1* exon. This is particularly significant given that exon 10b only encodes 1.7% of the open reading frame. Our results indicate that this exon should be implemented with priority in *NF1* mutation analysis.

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ORIGINAL INVESTIGATION

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RT-PCR splicing analysis of the *NF1* open reading frame

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Abstract Neurofibromatosis 1 (NF1) is an autosomal dominant condition whose molecular diagnosis is challenging because of the large size of the gene and the vast number of unique *NF1* gene mutations. Some splicing and nonsense mutations have been shown to cause exon skipping. Recently, temperature-induced abnormal splicing has been found in *NF1* in *ex-vivo* tissues. This prompted us to investigate the entire *NF1* transcript for such aberrant splicing. We found several novel exon skips that appeared de novo or were present initially and increased in aged/cooled blood: exon 20, exons 20 and 21 combined, exon 33, exon 34, exon 37, exon 40, exon 45, exons 43 and 45 combined, part of exon 43, and the first codon of exon 12b. Some aberrant splice forms were undetectable when blood was drawn into Qiagen PAXgene tubes, rather than EDTA vacutainers, and we demonstrate how these aberrant splicing events are a potential pitfall for RNA-based *NF1* mutation characterization. The same reverse transcription/polymerase chain reaction strategy was used to screen for novel *NF1* alternative splicing in Schwann cells and seven other tissues. Even though no Schwann-specific alternative exons were identified, we found minor novel splicing isoforms differentially expressed such as skips of exon 37 and exon 40. Skipping of exon 43, part of exon 43, and the first codon of exon 12b were found in all tissues analyzed. These forms suggest greater tissue-based variability in the *NF1* message than was previously thought and may indicate minor amounts of heterogeneity at the protein level.

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Introduction

Neurofibromatosis 1 (NF1) is a common dominant autosomal disorder with a frequency of 1/3500 worldwide. The major features include café-au-lait macules, Lisch nodules, and benign fibromatous Schwann cell tumors called neurofibromas (Friedman et al. 1999; Upadhyaya and Cooper 1998). The *NF1* gene is located on chromosome 17q11.2 and spans 350 kb with an open reading frame (ORF) of 8.5 kb (containing approximately 60 exons; Viskochil 1999). Exons 20–27a encode a GTPase-activating domain (GAP), which comprises the only region of neurofibromin whose biological function has been well characterized. Loss of heterozygosity in tumors, amongst other evidence, supports the two-hit hypothesis, indicating that *NF1* is a classic tumor suppressor gene in which both copies of the gene have to be mutated in Schwann cells to initiate neurofibroma formation (reviewed by Wallace and MacCollin 2001).

The *NF1* gene has a high mutation rate with most mutations predicted to lead to truncated proteins. The mutations occur throughout the gene and most have a frequency of less than 1% (Fahsold et al. 2000). The protein truncation test (PTT) is a popular mutation analysis technique for *NF1*, based on RNA, since it detects truncating mutations (Heim et al. 1995; Park and Pivnick 1998; Messiaen et al. 2000). However, false-positive PTT results clearly are a potential pitfall, especially if the test is not followed by characterization at the DNA level. Furthermore, many reported *NF1* mutations have not been characterized at the DNA and RNA levels or at the protein level, which is essential for fully understanding pathogenetic mechanisms.

NF1 is ubiquitously expressed, and three normal in-frame *NF1* splice isoforms have been reported. Exon 9br (30 bp) is specific to the brain (Danglot et al. 1995), exon 48a (54 bp) is muscle-specific (Gutmann et al. 1993), and exon 23a (63 bp), which alters GAP activity, is expressed in all tissues at various levels (Andersen et al. 1993). Additional "alternative" splice forms have been individually

reported, such as the out-of-frame skipping of exons 29 and 30, either alone or together in leukocytes, unrelated to NF1 mutations (Park et al. 1998). However, no transcript analysis of the complete *NF1* ORF in Schwann cells has been reported. Thus, one goal here has been to search systematically for Schwann-cell-specific isoforms, since this is the key cell type that is genetically abnormal and clonally expanded in neurofibromas (Muir et al. 2001; Rutkowski et al. 2000; Serra et al. 2000; Wallace et al. 2000).

Related to alternate/aberrant splicing, some nonsense *NF1* mutations have been found to cause the skipping of their corresponding exons, viz., 7 and 37 (Hoffmeyer et al. 1998; Messiaen et al. 1997; Wimmer et al. 2000a). Since these exon skips are in-frame (the coding sequence length is a multiple of three, and no stop codons are formed at the splice joint), the resulting protein could theoretically retain some function, missing only the residues encoded by that exon. Nonsense, missense, or silent mutations disrupting splicing enhancers in coding sequences have also been thought to lead to exon skipping in *NF1* and other genes (Liu et al. 2001; Shiga et al. 1997; Vockley et al. 2000). One recent study has suggested that a high percentage of *NF1* mutations causes altered splicing (Ars et al. 2000a). However, the majority of those mutations do not affect the conserved AG/GT dinucleotide donor and acceptors. Many of the splicing defects appear to induce exon skipping, whereas others utilize cryptic splice sites.

In addition, our group and others (Messiaen et al. 1999; Park et al. 1998; Wallace et al. 1998) initially identified a 31-bp insertion (from intron 4a) between exons 4a and 4b in some NF1 blood and tumor RNA samples. It has more recently been found that this insertion can be induced in less than 24 h in any leukocyte RNA by cold shock or ambient temperature, and the percentage of altered message tends to increase over time under cold shock (Ars et al. 2000b; Wimmer et al. 2000b). Together with the 31-bp insertion, two other altered *NF1* mRNAs are seen in this region: one lacks exon 4b, and one lacks exon 4b but has the 31-bp insert (Ars et al. 2000b). Additionally, the skipping of exons 7 and 37 has been found to occur under the same conditions (Messiaen et al. 2000); this is interesting in light of the nonsense-mediated skipping mentioned above (Hoffmeyer et al. 1998). These environmentally induced isoforms can lead to false-positive PTT results such as seen in Park and Pivnick (1998). Even an in-frame skip could cause a positive PTT test if the deletion is large enough, as with most exons. Such illegitimate splicing could potentially mask real mutations or lead to incorrect conclusions about mutation effects. These results from limited regions of *NF1* have prompted us to investigate temperature/age-induced splicing in *NF1* by leukocyte reverse transcription/polymerase chain reaction (RT-PCR) analyses across the entire ORF as a baseline for a comparison for mutation characterization. Our data are presented here.

Materials and methods

Samples

Total human RNA samples from brain, fetal brain, cerebellum, spinal cord, fetal liver, placenta, and heart were purchased from Clontech. Normal Schwann cell RNA was extracted from an anonymous non-NF1 Schwann cell culture by using Trizol (Invitrogen) as outlined in the protocol. For the initial RNA time/temperature study, two normal individuals and two NF1 patients gave blood under IRB approval. Leukocytes were isolated within 15 min by a red blood cell lysis procedure (Madison et al. 1987) and then immediately processed by using either RNAEasy (Qiagen) or Trizol. Blood aliquots were kept in EDTA vacutainer tubes for RNA processing at each time point: fresh, 24 h at room temperature, 3 days at 4°C, and 5 days at 4°C. For the study testing the PAXgene Blood RNA Validation Kit (Qiagen), blood from two unaffected individuals was drawn into these tubes and processed as outlined above (fresh, 24 h, 3 days). RNA was extracted as outlined by the manufacturer (Qiagen). It should be noted that "fresh" is actually at a 2 h time point after the drawing of blood, by which time all the blood cells have finished lysing.

RT-PCR and isoform characterization

Total RNA (1 µg) was reversed-transcribed by using random hexamer primers and Superscript II (Invitrogen) in a 20-µl volume. An aliquot of 1 µl cDNA was used as template for amplification in a Perkin-Elmer 480 Thermal Cycler with each primer set as follows: 1 min at 94°C, 1 min at the primer-specific annealing temperature, 1 min at 72°C for 38 cycles, and a 30 min final extension at 72°C in a 25-µl final volume by using *Taq* polymerase (Roche). Table 1 lists the primers, product size, and annealing temperatures. PCR products were separated on 8% native polyacrylamide gels (PAGE). Gels were silver-stained for highly sensitive DNA visualization as previously described (Abernathy et al. 1997). PCR products were shotgun subcloned into pCR 4-TOPO (Invitrogen). Clones containing alternative splice fragments were identified by PCR/PAGE and grown in 3 ml LB/ampicillin. Plasmid DNA was extracted by using the QIAprep Spin Miniprep Kit (Qiagen) and was employed as the template for cycle sequencing reactions with the ABI Big Dye Terminator and ABI Prism 310 automatic sequencer. Exon-specific primers were used for the sequencing of plasmid inserts.

Mutation analysis

Single-strand conformation polymorphism (SSCP) mutation analysis was performed in our laboratory by using conditions previously described (Abernathy et al. 1997) for the three NF1 patients in this report, resulting in the identification of the *NF1* exon 20 and exon 26 mutations. As described here, the exon 20 "mutation" is probably non-pathogenic, and the germline nonsense mutation was identified by PTT in patient RNA and confirmed in exon 16 DNA by Dr. Ludwine Messiaen with methods previously described (Messiaen et al. 1999, 2000). RT-PCR analysis to show cryptic splicing induced by the exon 26 mutation employed the conditions described above for the segment spanning exon 26.

Results

Time/temperature-induced splicing in NF1 and unaffected individuals

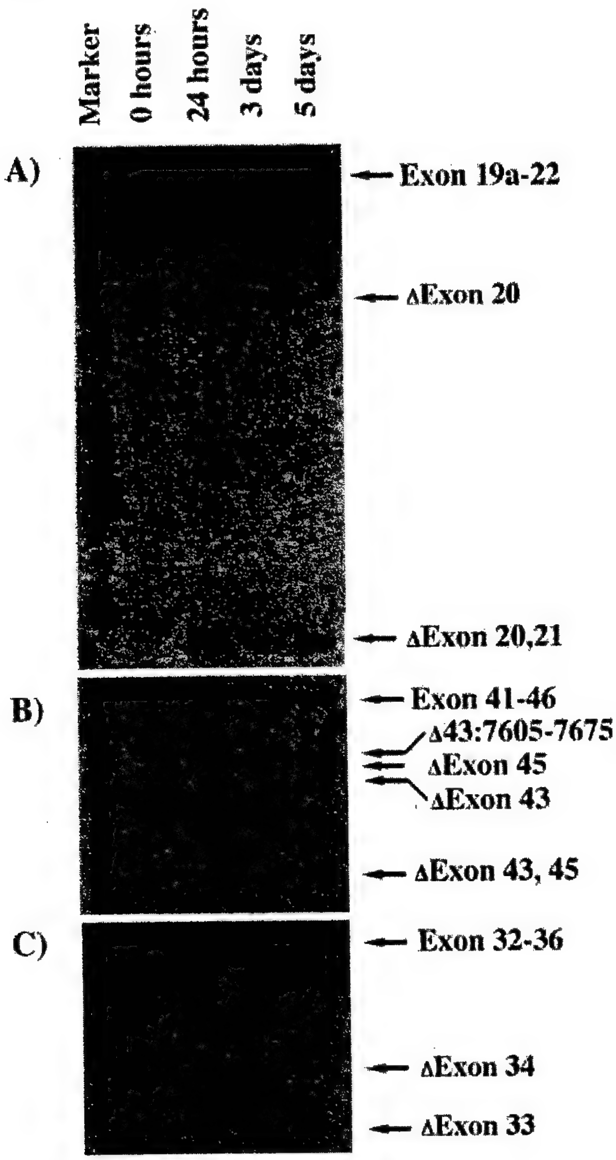
To test for the range of environmentally affected isoforms, we designed a complete *NF1* ORF splicing analysis with

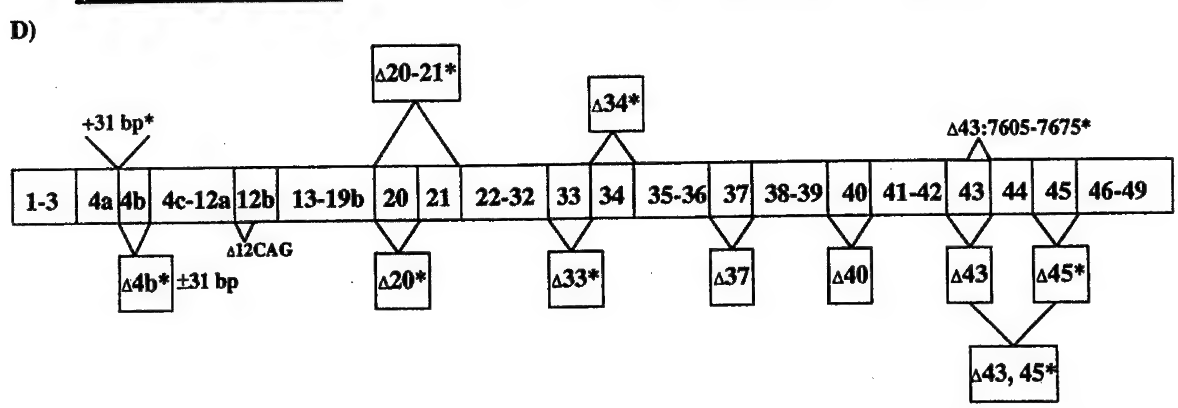
Table 1 RT-PCR NF1 primer sequences (*T** optimal PCR annealing temperature based on Perkin Elmer 480 thermal cycler)

Exon	Primer sequences 5' to 3'	Size (bp)	T* (°C)
1–5	GGACATGGCCGCACACAGG	689	53
	TCTGGATAATTTTCTACCCAGTTC	689	53
4a–8	AGCTGCAACAACTTCAATGCAGTC	658	65
	GAGAATGGCTTACTTGGATTAAA	658	65
7–10a	GGGAAGATAACTCTGTCATTTTCC	347	60
	GCACTGCTTTATGAAGTGTTTCAC	347	60
9–10c/11	CACCTTCTACATTTCACTATGTGC	456	60
	TGATGAAGAACCAGCAGAGCCTCC	456	60
10b–12a	AGTCTTACATTTAAAGAAAAAG	441	50
	GAAGAAATTTATTCCTGCAGATC	441	50
11–13	TCCTGATGCTCCTGTAGAAACA	547	55
	TGACAGAGGCAAACTCCATG	547	55
13–16	GATAGTGCAGCAGGATGCAG	428	55
	CTTGTGAAGGCTTTCAGCAGC	428	55
14–17	GAGTGATGGCACTGCTGAGC	608	50
	CAAATTGAGTATTGGTATCAGTC	608	50
16–19b	TTGAAGAATACCATCAGCAAGT	482	60
	TTCCATCAATTCCACACCAT	482	60
19a–22	TGGGTTATGGGAACATCAAACC	673	65
	TGCATGGAGTCTGCCAATTCTA	673	65
20–23–2	GTGCGCAAACAGGTGGCAGGAAACG	690	55
	GAGGAACTGATGATGGCATGGAAG	690	55
23–1–24	ATTGTGATCACATCCTCTGATTGG	302±63[a]	65
	ATCTAAAATCCCTGCTTCATACGG	302±63[a]	65
23–2–27a	TCAACTTCGAAGTGTGTGCCACTG	557±63[a]	60
	CTGGTAAGGTTAAGGCTGGACCAG	557±63[a]	60
26–28	TCCTACAAGTGATGCAG	640	55
	GGCCAGTCAGCAGCCGCTCATGAT	640	55
28–30	TCCTGGGTCAGGGAGTACACCAAG	599	65
	GTACAAGTTAAGGCACACAGAAG	599	65
30–33	[b]CATCGTCAGCTGCCTATAATCTTCTGTG	670	65
	[b]CATCTTGAAGGACAGCATCAGCATGTAG	670	65
32–36	[c]CATCGGATCCAGATCTGCTTGATGTTGTACTAG	749	60
	[d]CATCGTCGACTTGGTTGCAGGGATGGATTATATTG	749	60
35/36–41	GACAGAACTAGCTCAAAGATTTGC	693	65
	[e]CATCTTTCTATGTTTTAGGCTGCAGCGAC	693	65
40–42	[f]CATCGGTACAGGCATCCTTCACCTGCTATTG	428	65
	AAGCAACTTCTTAGTGTTGGCC	428	65
41–46	ATTCATCATGGTGACCCTTCCT	667	65
	GTTTGGTATTGTGGTGGGGATT	667	65
45–3'UTR	AATACTTAGCAGAGGCCAGTGTTGT	679	65
	GGGGAAAGGGCAAGGACAGGGAAG	679	65

[a]Size depends on inclusion of alternative splice exon 23a
[b]CATC addition to primer sequence
[c]CATCGGATC addition to primer sequence
[d]CATCGTCGA addition to primer sequence
[e]CAT addition to primer sequence
[f]CATC addition to primer sequence

RT-PCR primer pairs spanning exons 1–49, in 21 overlapping fragments (Table 1). We investigated samples from two unaffected individuals and two unrelated NF1 patients to check for any NF1-related differences, but none were found based on NF1 status, except for a single cryptic splice event attributable to the pathogenic mutation. This NF1 patient has a germline mutation in exon 26 (A4435G) leading to cryptic out of frame splicing (data not shown), whereas the *NF1* mutation in the other patient is not known and not evident from the RT-PCR analysis. Thus, the environment-sensitive alterations identified were consistent in all four samples and were unrelated to primary changes in the *NF1* gene. In addition to confirming the intron 4a 31-bp insertion reported previously (data not shown), we found environmentally affected splicing in 4 of 21 primer pairs, as described below and summarized in Fig. 1. Most of these isoforms appear to represent a small fraction of the *NF1* message based on silver-staining intensity. The exon 1–5 primer set often amplified additional bands, but these could not be reproduced with consistency other than the 31-bp insertion. The exon 19a–22 primer set revealed the induction of two lower bands consisting of the skipping of exon 20 and the combined skipping of exon 20–21, leading to a frame shift (Fig. 1A) in both cases. However, skipping of exon 20 ocurrs less frequently than the skipping of exons 20–21. The exon 41–46 analysis (Fig. 1B) showed four extra lower bands: two bands were the result of the skipping of exons 43 and 45, respectively, one band represented the loss of both exons 43 and 45, and one fragment lacked part of exon 43 (last 71 nucleotides; designated Δ43: 7605–7675, based on the traditional cDNA numbering





system). Exon 43 skipping was observed in all time point samples, although it inreased with time/cooling, and was the only one of the four bands that was an in-frame isoform. The exon 32–36 primer pair revealed two bands consisting of the independent skipping of exons 33 and 34 (Fig. 1C), both predicted to result in a frame shift. The skipping of exon 33 was observed in the time 0 sample and was not dramatically affected by time/temperature, whereas skipping of exon 34 was only present in trace amounts at all time points. The exon 36–41 primer pair showed two bands resulting from the skipping of exons 37 and 40; these were in-frame. Although both bands were present in trace amounts in the time 0 sample, skipping of exon 40 increased in the other time point samples (data not shown). In the exon 11–13 analysis, a lower band was present equally in all samples (including freshly processed RNA), and this ubiquitous novel isoform was found to be the result of the skipping of the first codon in exon 12b designated Δ12bCAG. This isoform would lead to a protein lacking Gln 616, a residue that is conserved in other species. The aberrant exon skips found in this study are shown diagrammatically in Fig. 1D. All of these splicing events had perfect exon/exon junctions at the sequence level, with the exception of Δ12bCAG, in which the AG of that codon was preferentially used as the splicing acceptor. Since we only amplified small regions at a time, it was unclear whether full-length transcripts would contain multiple isoforms. These aberrant isoforms showed de novo induction or an increase in intensity (i.e., ubiquitous) by 24 h; none appeared de novo after 24 h.

In addition, we collected blood from two unaffected individuals by using PAXgene Blood RNA tubes (Qiagen), which are advertised to contain reagents to stabilize and protect RNA from degradation. We applied the same methods as outlined above to test for the presence of aberrantly spliced isoforms in fresh (2 h as per protocol), 24-h, and 3-day-old samples to see if this system could prevent or minimize environment-related aberrant splic-

Fig. 1A–D Aberrant splicing in normal (non-NF1) aged blood, and overview of altered splice forms found. **A–C** Silver-stained 8% native polyacrylamide gels containing the 1-kb DNA ladder in lane 1 (Invitrogen), and RT-PCR products from blood samples of unaffected individuals processed at 0 h, 24 h, 3 days, and 5 days (lanes 2–5). *Arrows* Normal (*top arrows* in **A–C**) and aberrant splice forms. **A** Exon 19a-22 analysis. **B** Exon 41–46 analysis. **C** Exon 32–36 analysis. **D** Diagrammatic representation of all aberrant splicing, from both the temperature/time study and the tissue analysis. All exons in the NF1 transcript (not to scale) are indicated by corresponding *boxes*, and regions found to be individually spliced out in our study are shown *below* and *above* the transcript. The environmentally induced 31-bp insertion occurs between exons 4a and 4b. In exon 12b, the first codon is skipped (Δ12bCAG), and the last 71 nucleotides are missing in exon 43 (Δ43: 7605–7675). *Asterisks* Predicted frameshift with skipping of that exonic sequence; the other splice forms are in-frame

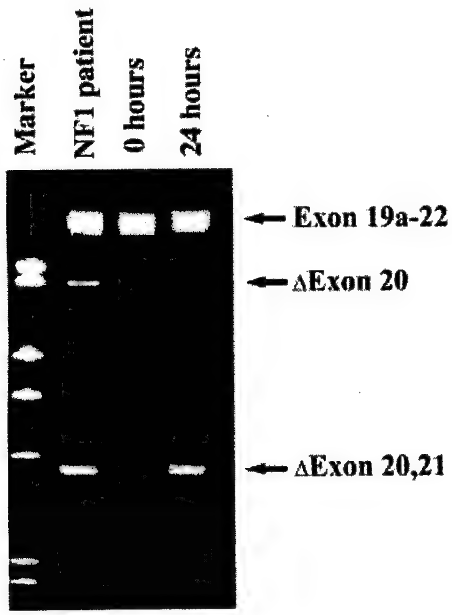


Fig. 2 RT-PCR Analysis of silent mutation in exon 20. Ethidium-bromide-stained native 8% polyacrylamide gel containing a 1-kb DNA ladder in lane 1, exon 19a–22 RT-PCR analysis of NF1 patient blood sample in *lane* 2 (patient with exon 20 silent substitution), and unaffected individual blood samples processed at 0 h and 24 h in lanes 3, 4, respectively. The Δexon 20 form is not seen in the fresh (0 h) sample and only in trace quantities at 24 h

ing. Based on this system, which lyses cells beginning immediately and is complete within 2 h, we expected and indeed observed no splice pattern differences between the PAXgene tube time points in either individual. However, some of the aberrant forms were still consistently present (data not shown): in the 36–41 primer set, the presence and quantity of the skipping of exon 37 and exon 40 was identical to that seen in fresh blood from EDTA tubes. Skipping of exon 43 and Δ43:7605–7675 occurred in the 41–46 primer set as seen in EDTA tubes, but skipping of exon 45 and exons 43 and 45 was not detectable with silver-staining. In the 32–36 primer set, only one band was present, viz., from the skipping of exon 33, as seen in EDTA tubes. The most profound difference was seen in the 19a-22 primer set, in that the skipping of exon 20 and exons 20–21 was not observed. Therefore, whereas the PAXgene blood tubes did not eliminate the presence of certain aberrant splice forms, there was a reduction in the overall number of such isoforms, and the system prevented the time-related accumulation of aberrant isoforms.

Environmentally induced splicing mimicking mutation-based illegitimate splicing

In another of our NF1 patients (and all affected relatives in the family), a novel silent mutation in a CpG dinucleotide in exon 20 (C3468T) was identified at the DNA level by exon SSCP. To determine whether this was pathogenic, via aberrant splicing, RNA was examined. In an RT-PCR based analysis of blood leukocytes (24-h-old), this mutation was associated with the skipping of exon 20 and exons 20–21 together, as shown in Fig. 2. As indicated in the previous section, skipping of exon 20 alone was found to be environmentally stimulated in all EDTA samples but only at a very low level. As can be seen in Fig. 2, the skipping of exon 20 in the mutant sample occurs at a greater frequency than in unaffected or other NF1 samples. Thus, until the time/temperature-induced splicing analysis was performed, it was theorized that the mutation might disrupt an exonic splicing enhancer and be the pathogenic NF1 mutation. However, a nonsense mutation in exon 16 was subsequently found via PTT (L. Messiaen, personal communication), and a tumor RNA sample from an affected nephew did not show increased exon skipping. This, together with our data from sample handling, suggests that the exon 20 mutation does not independently cause exon skipping and is coincidental and thus is not the pathogenic mutation despite initial appearances. However, Fig. 2 suggests that this variant might increase the tendency for an exon 20 skip, at least in leukocytes. Moreover, it should be noted that the skipping of exon 20 can be more readily detected in the 24-h normal sample in silver-stained gels (Fig. 1) than in ethidium-bromide-stained gels (Fig. 2). This illustrates the sensitivity differences between ethidium bromide and silver staining and emphasizes the need for high sensitivity systems to detect these isoforms, especially in freshly prepared RNA.

Survey of alternative splicing in Schwann cells and other tissues

The overlapping RT-PCR analysis, described above in fresh and aged leukocytes, was also used to assay for novel isoforms in Schwann cells. In addition to normal cultured Schwann cells, total RNA from seven other human tissues was included, in part as controls for known isoforms. In addition to finding the known isoforms in expected tissues, three primer pairs revealed novel isoforms in small quantities, as shown in Fig. 3 and summarized in Fig. 1D. The exon 36–41 primer set showed two lower bands predominantly in Schwann cells and heart (Fig. 3A). One of the lower bands was also present in the brain and spinal cord samples. The upper band was also faintly present in fetal liver. Sequencing of the isoforms revealed that the top abnormal band represented the skipping of exon 37, and the bottom band represented the skipping of exon 40. The exon 41–46 analysis revealed two lower bands in all tissues and was the result of the skipping of exon 43 and part of exon 43 (Δ43:7605–7675), respectively (Fig. 3B). Exon 43 is 123 bp and so skipping of the whole exon predicts an in-frame deletion, whereas the isoform lacking the last 71 bp would be out-of-frame, utilizing a cryptic acceptor. In addition, the exon 11–13 primer set shows one lower band present in all samples. This is the same isoform identified in the temperature/time-induced splicing study and is shown in Fig. 3C (Δ12bCAG).

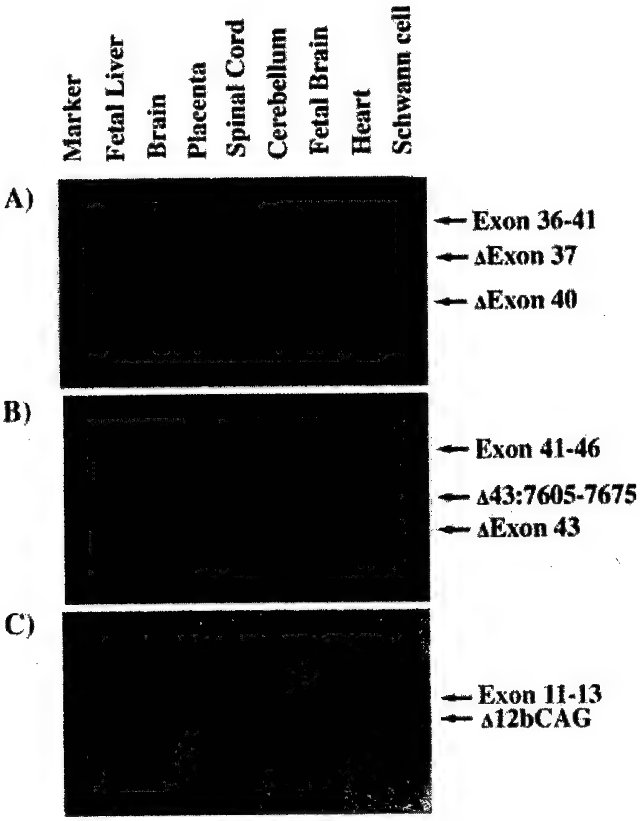


Fig. 3A–C NF1 alternative splicing analysis in various tissues. Silver-stained native 8% polyacrylamide gel containing a 1-kb DNA ladder in lane 1. RT-PCR products from various tissue types are indicated *above* each lane. **A** Exon 36–41 analysis. **B** Exon 41–46 analysis. **C** Exon 11–13 analysis showing skipping of the first codon in exon 12b (*Δ12bCAG*). *Arrows* Normal and aberrant splice forms with the expected normal fragment at the *top arrow* in **A–C**

Discussion

We report novel aberrant *NF1* splicing, which is not random and affects only specific parts of the transcript across its length. We do not know how many of the isoforms might be included in a single transcript, which makes quantitation difficult; however, most of these forms individually seem to represent less than 10% of the RT-PCR product, depending on the age of the sample. Nevertheless, it is possible that if these occur on separate molecules, then overall, a significant portion of the NF1 transcript could be corrupted, especially in older samples. Whereas most isoforms appearing in the 24-h and older samples show induction over time, some also can be seen in the fresh samples (usually at lower quantities) and therefore might represent isoforms that are present in vivo or that can be very quickly environmentally induced. Although there were no significant differences between which isoforms were observed in samples from NF1 patients and unaffected individuals, the intensity of each isoform varied somewhat between samples, consistent with environmental factors playing a role in the overall expression of these isoforms. Since most of the aberrant exon skips are in-frame outside the GAP domain, any resulting proteins from such isoforms might be stable and have normal GAP activity, the two major exceptions being the 31-bp insertion (out-of-frame upstream of GAP domain) and the exon 20–21 skip (out-of-frame and within the GAP domain). Most constitutional *NF1* mutations are predicted to result in truncated proteins, but this has not been conclusively tested and reported at the protein level. The 31-bp insertion (and forms lacking exon 4b) occurs on both *NF1* alleles in patients and unaffected individuals (Wallace et al. 1998) and thus is not attributable to sequence alterations in the *NF1* gene. These and many of the other isoforms reported here and elsewhere are the result of environmentally stimulated aberrant splicing in *ex-vivo* leukocytes related to time and temperature. The PTT is the most widely used test to identify potential *NF1* mutations and is often based on RNA analysis of blood that is typically 18–36 h post-draw. In at least one reported case, an abnormal PTT (positive test) was found to be caused by the 31-bp insertion, and this phenomenon poses a risk of false-positives in any of the PTT segments based on our finding of multiple affected exons across the gene. Therefore, it is prudent for positive PTTs or aberrant RT-PCR analysis to be confirmed at the DNA level in order to establish that a pathogenic *NF1* gene mutation exists at that site.

Alternative splice-derived isoforms of proteins are known to be important in development and tissue-specific functions. Novel isoforms (both in- and out-of frame) could provide important clues to neurofibromin functions, particularly in neural-crest-derived tissues. However, no comprehensive ORF RT-PCR analyses in Schwann cells has been reported. Since the three known *NF1* alternatively spliced exons (9br, 23a, 48a) are very small (30–63 bp), small overlapping RT-PCR segments (350–800 bp) and PAGE analysis were designed to visualize small differences for scanning the *NF1* gene. Thus, our ORF analysis was very thorough, and we detected minor splicing events not previously reported. These all represent full (perfect) or partial exon skips and not the identification of new exonic material. The use of commercially prepared RNA (obtained from tissues frozen immediately upon arrival) and Schwann cell RNA rapidly extracted from fresh cultures minimizes the chance that these RNA samples contain the time/temperature RNA artifacts above. Furthermore, not always the same tissues showed these variants, and the results were reproducible with different cDNA preparations. However, the consistent skipping of Δ12bCAG, exons 37, 40, and 43 was found in both parts of our study and in the PAXgene tube samples (which represented cells lysed within 2 h of being drawn), suggesting that these events may be present at a low level in vivo (possibly tissue-specific) or that they can be environmentally induced within a very short time ex vivo. In each case, the isoform does not contain a frameshift and thus it is feasible that stable proteins might result. Moreover, none of these skips (37, 40, 43, and Δ12bCAG) involves the GAP domain. This leads to the prediction that any resulting proteins would not have altered GAP activity.

Mutations leading to stop codons in exon 37 have been reported to be involved in exon skipping. However, other nonsense/frameshift mutations in exon 37 do not cause skipping (Colman et al. 1997; Hoffmeyer et al. 1998; Robinson et al. 1995; Upadhyaya et al. 1997). Further analysis have revealed that certain mutations predict alterations in the RNA secondary structure; these might be the cause of exon 37 skipping (Hoffmeyer et al. 1998). Stress conditions, such as cold shock, could lead to alterations in the secondary structure of RNA in certain regions of the molecule and might be responsible for the exon skipping in aged blood samples. The same has been shown in another study of exon 7 skipping in which the RNA structure overall was not affected by mutations, suggesting that the skipping of exon 7 can be easily induced or that the mutations disrupt exon enhancer sites as an alternative mechanism (Wimmer et al. 2000a). Wimmer et al. (2000a) have also found 11 different RT-PCR aberrations within exons 1–10a by using aged blood of a normal individual, showing that this can mimic results from alterations of exon splicing caused by mutations in NF1 patients (Wimmer et al. 2000b). However, other than the exon 7 observation, we have not clearly detected any of their other reported abnormalities suggesting that laboratory-specific methods and factors may also play a role in the precision of these results. Furthermore, our observations raise questions about the reported nonsense-related exon 7 and 37 skips. The issue of whether these mutations do cause skips or partially contribute to them will be important to address, since the pathogenesis of a nonsense mutation is potentially much different from that of a transcript missing a minor fraction of codons (which would not be subjected to nonsense-mediated decay). Therefore, it is also important for RNA mutation analysis to control for such spurious events by using fresh cells, e.g., cultured lymphocytes or fibroblasts (Messiaen et al. 2000) and processing them promptly.

An example of how a mutation characterization can be erroneous was shown by our novel silent mutation in exon 20 (C3468T); the RNA sample showed aberrant splicing around this exon. However, since tumor RNA from another family member did not show the increase in exon 20 skipping, and a clear nonsense mutation was found elsewhere, it is likely that the exon 20 substitution is a rare neutral variant. It is possible that this point change might affect an exonic enhancer, boosting the propensity for illegitimate splicing in this region, at least in leukocytes. This example illustrates the complexity of RNA-based mutation characterization, especially if samples are not fresh or are from different tissues processed under different conditions.

Since the novel isoforms that we have identified in different tissues are generally poorly represented, it is possible that some are environmental artifacts despite rapid sample handling. However, freshly processed RNA and the PAXgene tube study suggest that at least some isoforms might represent true in vivo phenomena, i.e., ubiquitous, perhaps with specific roles, or that NF1 splicing has a certain level of inherent error that may or may not be exacerbated by stressors. Although effects of only time and temperature have been studied thus far, they set the precedence for the involvement of other factors that could contribute to the regulation and maintenance of NF1 transcripts, including in vivo effects. If such effects lead to a significant reduction in full-length neurofibromin production, i.e. a somatic epigenetic negative effect, it could contribute to the NF1 phenotype. Awareness of these phenomena is important for future studies of the *NF1*/neurofibromin protein, splicing metabolism (in general), and interpretations of RNA-based mutation analyses.

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NF1 Mutations and Molecular Testing

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ABSTRACT

Neurofibromatosis 1 (NF1) is a progressive autosomal dominant condition caused by mutations in the *NF1* gene on chromosome 17. The condition shows clinical variable expressivity, with varying features even between family members who share the same mutation. Furthermore, it is impossible to precisely predict the severity and course of the condition, a source of frustration for families and physicians. NF1 is also heterogeneous at the mutation level, with over 300 independent mutations having been reported in this gene. The mutation data have accumulated slowly due to the variability of the mutation types and the size and complexity of the gene. This is also reflected in the lack of a simple, inexpensive, highly accurate DNA-based test for NF1 at present. This article reviews current *NF1* mutation spectrum and testing, discussing and illustrating mutation mechanisms and pathogenetic effects, as well as factors affecting DNA testing and interpretation/diagnosis.

Background about NF1. Neurofibromatosis 1 (NF1) is among the most common autosomal dominant medical conditions worldwide, with an estimated incidence of 1/3500 in all ethnic and geographic-based groups.[1] In part, its prevalence and lack of ethnic predilection seems to be due to the fact that about half of newly-diagnosed patients have no family history of NF1, suggesting that new mutations are frequently occurring. One of the major features is the development of benign neural crest tumors, mostly neurofibromas, although a number of other non-tumor features may be present as well. This condition manifests in a unique fashion in every patient, with respect to the exact type and timing of individual features, even within monozygotic twin pairs and within families carrying the same mutation. This variable expressivity has led to adoption of a diverse set of clinical diagnostic criteria which is considered the gold standard for NF1 diagnosis, as discussed elsewhere in this proceedings (J.M. Friedman). Despite this, however, many young children and some older patients fail to meet diagnostic criteria and are in limbo until the future, needing regular follow-up to see if further features appear that would confirm the diagnosis. For these patients, and for families who desire prenatal diagnosis, a DNA- or blood-based conclusive test would be helpful.

NF1 Mutation Detection Difficulties. Toward that goal, the *NF1* gene was cloned in 1990 and over the next several years its gene structure was identified.[2-5] As described elsewhere in this proceedings (D. Viskochil), the gene has 60 exons scattered over 350 kb of genomic DNA on chromosome 17q11.2. *NF1* germline mutation analysis has been difficult and labor intensive due to not only the size of the gene but also to the following factors: (1) mutations have been found in nearly every exon with most being novel and few recurring more than 1%, (2) the presence of the normal allele masking mutation, (3) the presence of homologous loci on other chromosomes which co-amplify with many of the

exons in the 5′ half of the gene, (4) reduced RNA levels of the mutant allele due to nonsense-mediated decay can confound RNA-based detection, (5) many of the mutations appear to be intron changes affecting splicing, (6) known RNA exon skipping artifacts which occur in blood or other cells whose RNA is not processed immediately, and (7) the fact that 5-10% of mutations are microdeletions spanning the *NF1* gene, which escape detection by standard PCR-based exon or RNA-based analyses. Thus, mutation data have accumulated slowly since 1992, and are scattered among a large cohort of publications (in part reviewed by Upadhyaya and Cooper, 1998).[6] An attempt to catalog the known mutations was pursued by a National Neurofibromatosis Foundation-sponsored International Mutation Database until early 1999 (mostly relying on voluntary submission), which is a fairly detailed and accurate holding up to that point (www.nf.org). Several publications since then, however, have reported data from long-term studies which applied fairly extensive analysis to large patient sets; in particular, three report the largest sets of data: Messiaen et al., 2000; Ars et al., 2000; Fahsold et al., 2000.[7-9] It is clear from all of the literature and the Mutation Database that nearly all *NF1* germline mutations are predicted to be deleterious at the protein level, either resulting in a null allele or a predicted truncated protein with subsequent altered properties (if such a protein is even stable). Some missense mutations have been reported, some of which clearly disrupt functionally-important amino acids. The papers listed above, as well as other literature, suggest some possible patterns in the mutation data that might indicate which exons are more frequently mutated (e.g. exons 4a, 4b, 7, 9, 10a, 10b, 10c, 11, 16, 23-2, 29, 31, 37, 42, 45), or the most common mechanisms. Such data can help guide future mutation detection development.

NF1 Mutations and Mechanisms. The mutation mechanisms, therefore, fall into several categories, which are described below with some examples shown. Frequencies

reported here are compiled from the recent literature and the Database. One mechanism is DNA replication errors, in which the mutation may be the loss or gain of a small direct repeat sequence within the open reading frame; these are likely due to slippage during replication, or the deletion of a sequence which has inverted repeats at the ends and is thus looped out as a hairpin and skipped in replication. The literature and the Mutation Database together suggest that the frequency of this type of mechanism is 20-30%, and usually results in a deletion rather than an insertion. One key in interpretation is whether the error is in-frame: if it involves a multiple of 3 bases, then the resulting protein could be simply missing amino acid(s) (or have additional ones), or if the error occurred in the middle of a codon, then the resulting in-frame shift might actually lead to a premature stop codon downstream. If the error is out-of-frame then the clear prediction is early termination, usually within 20 amino acids. The issue of whether truncated NF1 proteins (neurofibromin) are stable and have any function has not been completely addressed.

Another mutation mechanism seen in NF1 is point substitutions (single base changes that are not silent). Specifically, point substitutions which create stop codons (nonsense mutations) have a 30-38% frequency, while other point changes in the open reading frame are predicted to alter the amino acid encoded by the affected codon; these missense mutations appear to occur 5-10% of the time. However, predicting the pathogenetic mechanism of these mutations is far from straightforward, since it is becoming apparent that some of these substitutions actually affect RNA splicing (see examples ahead).

Mutations which clearly affect splicing (e.g. altering the consensus splice donor or splice acceptor sequence, usually causing the skip of the nearby exon) appear to occur about 20-35% of the time. However, since many mutations have been reported at the DNA level without RNA level analysis, it is possible that splicing related mutations (skips or

cryptic donors/acceptors) might represent the actual mechanism in a larger-than-expected proportion of mutations. For example, our laboratory recently detected a novel exon 26 mutation that would appear to be a missense mutation (A4435G, Ser1479Gly). However, our RT-PCR analysis across exons 24 to 29 showed that this mutation actually introduces a cryptic splice acceptor, resulting in skipping of the first 68 nucleotides of exon 26 and an ultimate frameshift (Figure 1). Another example of this is a recurrent mutation in exon 10b (A1466G) which, rather than causing a missense, also induces cryptic splicing leading to a frameshift.[10] A few other examples have been reported elsewhere, which emphasizes the importance of characterizing mutation effects at the transcript level as well.[7,8]

Other *NF1* mutations include large intragenic deletions and insertions, which together appear to represent less than 5% of the cases, although these can be difficult to detect with standard methods and so may be under-represented in the literature (e.g. 90 kb deletion; 12 kb deletion)[11,12]. The other rare unusual mutation class consists of chromosome translocation or inversion disrupting the gene; a few cases have been reported (reviewed by Upadhyaya and Cooper, 1998)[6].

Finally, it is thought that 2-10% of all NF1 cases have a large deletion encompassing the *NF1* gene plus surrounding sequences, a 1.5 megabase deletion mediated by direct repeats.[13,14] These patients seem to be at increased risk for early and greater tumor burden, as well as mental deficits and dysmorphism. These large deletion cases have been particularly valuable in revealing mosaicism in NF1, the finding that some patients have unaffected cells, presumably due to somatic mutation early in embryogenesis. In particular for the large deletions, the mechanism in somatic tissue would be intra-chromosomal recombination followed by excision of the intervening material; whereas in the germline, unequal inter-chromosomal recombination would be the mechanism leading to a gamete lacking this region. Of interest is the fact that these mutations are almost

always of maternal origin. In contrast, most new mutations are of paternal origin, the vast majority of which are small intragenic mutations, although it is unclear if there is an increased risk of *NF1* mutation in spermatogenesis with advanced paternal age.[15,16]

Most Recurrent Mutations. The *NF1* mutation data have accumulated sufficiently to see some patterns of recurrence of small mutations. The most common ones are mentioned here; these may be important in efforts to draw phenotype/genotype correlations. Although it is difficult to accurately estimate the frequency of these recurrent mutations, it is clear that none are more than 2-3%, most less than that. The first reported and best-known recurrent mutation is the exon 31 nonsense mutation R1947X, caused by a CpG C to T transition, which appears to have a frequency of about 1% (Dublin et al., 1995; Andrews et al., 1996; Wallace et al. unpublished data: 3/240 independent cases).[17,18] Exon 10b appears to be a target for mutation (perhaps containing 3-4% of germline mutations), with a recurrent cryptic-splice-inducing point change and a 4 bp deletion reported.[10] Exons 10a and 10c may have increased mutation rates, too, with exon 10a having recurrent stop mutations R440X and R461X.[7] Also, exon 37 has a relatively high rate of mutation, including recurrent mutations 6789delTTAC and Y2264X (reviewed by Upadhyaya and Cooper 1998; Messiaen et al., 2000, Fahsold et al., 2000).[6,7,9] Exon 4b also has recurrent mutations 499delTGTT (about 2% frequency reported[19]; our lab has found that mutation in 1.7% of patients (3/173), unpublished data), and D176E.[9,19]

Hints of Phenotype/Genotype Correlations. Clinical data suggest that there is less variation within a family than between families, and also support the notion that other genes play minor roles in NF1 phenotype, which predicts that studies may derive some correlations between mutations (at *NF1* and other loci) and features.[20] A few specific publications support this idea, although the data are sparse and it remains to be seen if

these findings hold up with greater numbers. For example, a family with only café-au-lait spots appears to show linkage of this trait to the *NF1* gene region, despite having no other signs of NF1.[21] A small study recently suggested that large *NF1* deletions are more common in patients who develop malignancies such as MPNSTs.[22] Also, a stop mutation in exon 37 was found in a patient who, at age 21, fails to meet NF1 diagnostic criteria and yet does not appear to be mosaic.[23] In another example, all 5 affected members of a family with an exon 46 frameshift mutation consistently showed spinal neurofibromas and café-au-lait spots, with other features only variably found.[24] We had a similar experience with one de novo patient in his 30s who had spinal neurofibromas but only one or two detectable dermal nodules.[25] There are also a few reports about *NF1* gene mutations in patients who fit other syndrome diagnoses better, which suggests that different clinical disorders might be allelic: two reports of families/patients with Watson syndrome (which has some overlapping features) having *NF1* gene mutations,[26,27] and a patient with LEOPARD syndrome.[28]

<u>Current Methods of DNA/RNA-based NF1 Diagnosis</u>. There are two basic categories of molecular diagnosis of NF1: linkage analysis and direct mutation detection. Linkage analysis tests for co-segregation of the disease allele with particular marker alleles in a family, in order to identify which chromosome 17 carries the *NF1* mutation (usually for a prenatal diagnosis). This is very effective in familial cases of NF1 because of the numerous highly informative markers in the gene, and strong linkage disequilibrium.[29,30] Together, these factors help ensure that a parent's mutation-carrying chromosome can be identified, and that the risk of crossover confounding a linkage prediction is virtually nil. Thus, in cases where it is applicable, linkage analysis is very powerful and accurate in NF1, although it can never be considered 100% certain. Figure 2 shows a hypothetical pedigree with a marker genotype from a microsatellite polymorphism in the *NF1* gene.

This shows clear segregation of the disease allele with the "5" allele, indicating that these are in cis and that tracking of the "5" allele from an affected individual will be informative for prediction of passing the *NF1* mutation to offspring. Note that in other families, different microsatellite alleles may be linked to the mutation. NF1 linkage analysis can theoretically be applied in a family with only one affected individual as long as there is at least one offspring whose phenotype is very clear. Thus, in Figure 2 if you were only able to ascertain the couple II-2/ II-3, who had only their first child (clearly unaffected) but were interested in prenatal diagnosis for future children, the linkage information from that first child alone would suggest that the 3 allele transmits with the normal *NF1* gene. Having data from additional family members (i.e. more meiotic events) helps strengthen the conclusion and is preferable. An advantage of linkage analysis is that the actual disease-related mutation does not have to be identified; weaknesses include the slight risk of wrong conclusion (less than 1% in a family with informative polymorphisms at both ends of the gene and internal markers), and the fact that multiple family members have to participate.

Since direct *NF1* mutation analysis has still mostly been under scrutiny in research labs, there are only a few clinical labs in the world doing direct detection, using a variety of means but mostly based on the protein truncation test (PTT). This test studies the *NF1* transcript through a series of overlapping RT-PCR products amplified with a 5′ primer containing transcription and translation signals. Thus, a fragment of the open reading frame from a patient's *NF1* genes can be amplified and subjected to *in vitro* transcription/translation using radiolabeled amino acids, to force the RNA to reveal whether it carries a truncating mutation. The labeled proteins are separated on polyacrylamide protein gels, and autoradiography reveals whether there are any fragments below the normal size that might suggest a truncating mutation (see Figure 3

for example). Since most *NF1* mutations do encode protein-level truncations, this is a fairly informative test for the effort, although it is reported to only have a 53-73% sensitivity.[31-33] This test would be expected to detect nonsense and frameshift mutations, the latter of which could occur through insertions/deletions or via aberrant mRNA splicing. Mutations resulting in proteins only slightly shorter or longer, or very short, might not be detected due to gel resolution; our lab tries to circumvent this pitfall by running two gels—one short to resolve small fragments and one long to resolve the fragments closer to full length. The false-positive and false-negative rates have not been precisely determined, which is another pitfall.

The success of the PTT method is affected by several factors. One is that many mutant RNAs are decreased in quantity to various levels, the result of which is that a positive smaller PTT band would be fainter than the normal and might escape detection. Likewise, the RNA must be high quality to produce adequate quality and quantity of RT-PCR products, or the analysis will fail or be susceptible to PCR-based artifacts. A related problem is that the *NF1* gene is known to undergo spurious exon skipping in response to environmental stress, as in time/temperature after blood draw.[8,34,35] This has the potential for causing abnormal truncated PTT bands. A control for that artifact would be to confirm a PTT-based mutation at the DNA level, although this is not always straightforward if a splicing error is involved, and not all commercial labs do this. Another option to prevent that situation would be to process tissue/cells/blood immediately for RNA, and/or employ short-term tissue culture to "refresh" the cells and generate new cells through mitosis.

The 1.5 Mb microdeletions are best detected by fluorescent in situ hybridization (FISH), a molecular cytogenetic technique, although again not many clinical cytogenetics labs offer this as a commercial test at the moment. Furthermore, while some patients

might be suspected of having such a deletion due to their phenotype, large deletions have also been found in patients who do not have overtly severe features.[36] Thus, it is not always clear when to order the FISH test, but one lab has rolled this analysis into a multi-method approach (PTT, FISH, heteroduplex analysis, Southern blot) to yield a 95% detection of *NF1* mutations in their test set of 67 samples.[7] As an alternative, the large deletions can be detected by genotyping of the nuclear family with *NF1* polymorphisms, provided that the markers are sufficiently informative to see lack of a parental allele, although this is not as amenable to commercial application as the FISH test.[36]

There is no single comprehensive source listing of all labs doing *NF1* molecular testing, although the publicly funded website at GeneTests (www.genetests.org) accepts listings from commercial and research labs and provides that information to healthcare providers at no charge. Once a specific *NF1* mutation has been detected, a fairly simple test can usually be devised to screen for that mutation in other family members or other NF1 patients/population, such as the restriction digest system shown in Fig. 1 for the exon 26 mutation. PCR-RFLP tests that rely on gain of a restriction site are very specific. However, PCR-RFLP tests that are geared to detect loss of a restriction site due to a mutation have a minor pitfall in that one might misinterpret partial digestion as a mutation; furthermore, any base alteration within the enzyme's sequence will ruin the restriction site and thus look identical on a gel (and so should be followed up by sequencing if you are trying to track a specific mutation among different families).

The Issue of Pathogenicity. The worst case scenario for NF1 molecular diagnosis is to fail to find linkage in a family or a mutation in a patient/family. There are no other options for such patients except to rely strictly on diagnostic criteria, often a waiting game. The next worse scenario is that a mutation is detected, but its pathogenicity is unclear, such as missense mutations. Outside of the GTPase-activating domain of neurofibromin

(GAP, encoded by exons 21-27a), there are no assays for functional effect of a missense. It would be recommended that the region spanning the putative mutation be examined for RNA-level abnormalities such as splice errors. Also, the mutation is more likely to cause a change in amino acid group (non-conservative change) in the pathogenic case. Furthermore, a lab could test whether this alteration is only found in affected individuals in the family or also within a sample of at least 50 unrelated unaffected individuals (preferably of the same ethnic background). These would help test the hypothesis that this change is a neutral rare variant, and not the NF1 disease-related mutation. However, if a lab can show that this change is linked to the mutant allele, then it becomes informative as a linkage-type system. A final piece of evidence would be to determine whether the affected amino acid is conserved in other species, which might indicate a strong functional role for that specific residue. If there is any question about the mutation being pathogenic, this must be taken into consideration for diagnosis or genetic counseling. In such instances it would be helpful to do as complete an analysis as possible in case another (more clearly pathogenic) mutation could be found. There are at least 2 cases of NF1 patients with two independent gene alterations; it is unknown whether one or both are related to the disease.[9]

Clinical Indications. At the moment, DNA-based testing is unnecessary to make a diagnosis if the clinical diagnostic criteria are clearly met. However, it could be implemented to test individuals who do not meet diagnostic criteria but have suspicious findings, or to implement prenatal testing (with the caveat that the current state of the art in NF1 testing might not be able to help some individuals). Detection of the specific mutation does not yet yield any additional information of clinical help in management or prediction of severity/progression. However, it is possible that such genotype/phenotype correlations will be identified in the future, at which time detection of the specific

mutation may be of significance. Physicians should also be aware that molecular testing of new-mutation cases runs the risk for a false-negative test if that individual is mosaic and the tissue sent for testing does not contain a high proportion of affected cells. Thus, for any patients considered possibly mosaic, one option would be to test clearly-affected tissue such as a tumor or café-au-lait spot fibroblast culture. Outside of the issue of diagnosis, health care providers interested in helping contribute to derivation of genotype/phenotype correlations could provide detailed clinical information and any DNA-based information to NF1 databases such as maintained by Dr. Jan Friedman (Univ. of British Columbia, Vancouver).

Tumor Mutations. NF1 tumors follow the Knudsen two-hit tumor suppressor model at the *NF1* gene, including both benign and malignant nerve sheath tumors (reviewed by MacCollin and Wallace, 2001).[37] Thus, an originator tumor cell will have contained the germline mutation like all cells in that body, but will also have suffered mutation of the remaining normal allele, resulting in a cell with no normal functioning neurofibromin. These somatic mutations appear to cover a wide range of mechanisms, from loss of heterozygosity essentially deleting part or all of the *NF1* locus and flanking regions, to specific small mutations in the *NF1* gene. At current time there is no clinical reason to do an *NF1* mutation test on tumor material from an NF1 patient, although it is possible that testing of an isolated tumor in a patient who otherwise shows no signs might be useful to help rule out whether that person has a germline or mosaic *NF1* mutation. However, it is possible that in the future, with improved research about pathogenetic mechanisms of *NF1* mutations and downstream effects, that genotyping of tumors might be indicated to help design therapies specifically to treat those tumors.

Future Prospects. *NF1* mutation analysis will certainly become much more sensitive and straightforward in the near future, with implementation of newer methods

and equipment designed for large gene/multiple mutation analysis such as BRCA1 using an Affymetrix oligonucleotide chip.[38] The detection will become simpler, based on accumulated data about best recurrent mutations/exons/mutation types, and perhaps using systems such as denaturing HPLC, although the issue of pathogenicity will always be important.[39,40] Therefore, pathogenetic mechanism should continue to be investigated for all mutations, on the hypothesis that genotype/phenotype correlations may be based on the RNA/protein level effects, which would lead to clinically-useful information. It is also possible that blood protein-based tests may be developed as another form of NF1 marker; for example, it has been found that NF1 tumor cell and skin fibroblast cultures secrete midkine, a protein normally down-regulated in nearly all adult tissues but known to be associated with tumors.[41] However, the accuracy of the clinical diagnosis is extremely good, and so even if a simple/cheap DNA or blood tests is available, there will not necessarily be a need to order it only for the purpose of diagnosis, especially for straightforward cases. Also, the future will likely reveal that variants at other genes help dictate NF1 phenotype (so-called modifying genes)[20], and so it may be that patients are tested for these other genes to help guide medical care.

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FIGURE LEGENDS

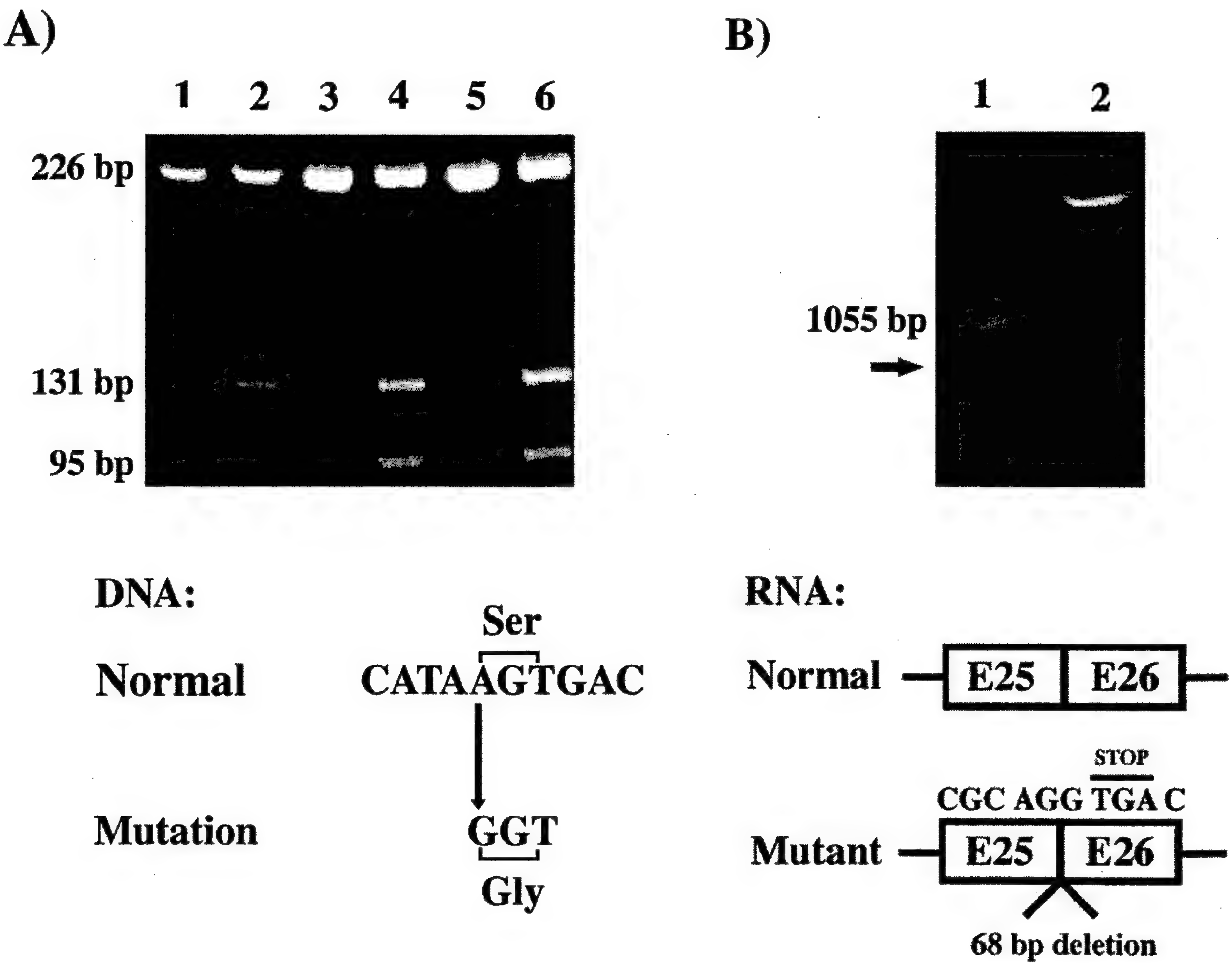
<u>Figure 1.</u> (A) shows an ethidium bromide stained gel of exon 26 PCR products digested with HphI and separated by electrophoresis on an 8% native polyacrylamide gel. The mutation detected and sequenced in NF1 patient UF450, illustrated below the gel, coincidentally creates a novel HphI site which can be conveniently assayed by PCR amplification of exon 26 followed by HphI restriction enzyme digestion and separation of fragments by gel electrophoresis. Lanes 1, 3, and 5 show the normal *NF1* gene HphI pattern (no HphI cuts). Lane 2 shows the pattern from UF450 leukocyte DNA, lane 4 shows the pattern from UF450 plexiform tumor DNA, and lane 6 shows the result from DNA extracted from a Schwann cell culture of the same plexiform tumor. In lane 2, both the normal allele (top band) and the mutant allele (represented by both lower bands) are evident as expected for constitutional DNA in a heterozygous condition. The heterozygous pattern in lanes 4 and 6 suggest that the tumor and its cell culture are also heterozygous at this site, indicating that the somatic *NF1* mutation resides at another site on the other allele and is not likely a large deletion. Exon 26 amplification and analysis was done using methods/primers/conditions described previously.[42]

(B) shows an ethidium bromide stained native polyacrylamide gel separating RT-PCR products spanning exons 24-29 from UF450 (lane 1). Lane 2 contains the Invitrogen 1-kb ladder for a marker standard (1 and 1.6 kb bands shown). The patient's sample reveals the normal expected RT-PCR product (1055 bp) as well as an abnormal product below that (arrow). Sequence analysis of the RT-PCR product showed that the mutant cDNA is missing the first 68 bp of exon 26 due to creation of a cryptic splice acceptor by the mutation, as illustrated below the gel. This frameshift results in an immediate downstream stop codon and would be predicted to lead to a truncated protein. RT-PCR

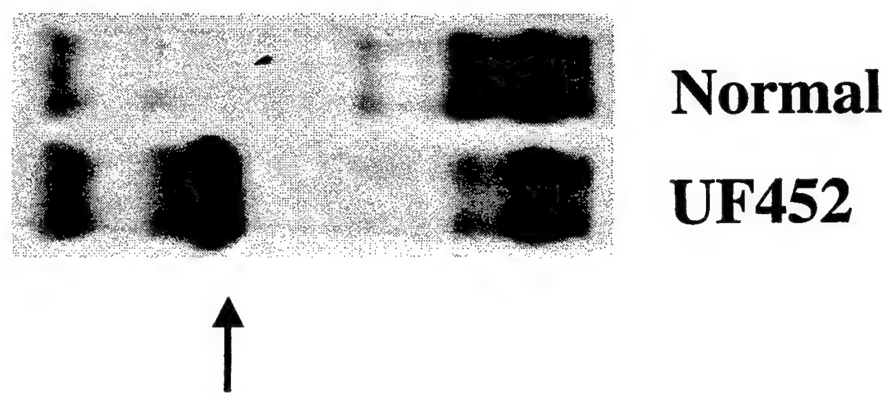
primers were PTT-24F (5'-GGATCCTAATACGACTCACTATAGGGAGACCACCATGGAAGCAGGGATTTTAGAT), and PTT-3R (5'-TRGGACTTTTGTTCGCTCTGCTGA); the method employed Invitrogen's Superscript II reverse transcription system. PCR and sequence analysis was done as previously described, using automated sequencing.[42]

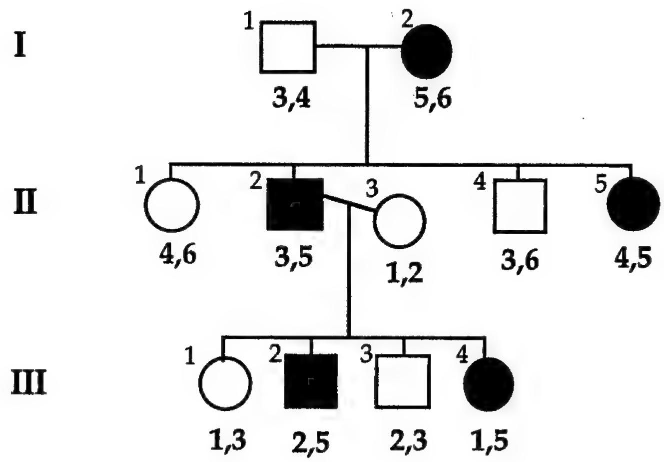
Figure 2. A hypothetical three-generation NF1 pedigree is shown, with squares representing males and circles females. Darkened symbols represent affected individuals. Generation numbers are listed to the side in Roman numerals, and individual numbers are in Arabic to the upper left of each symbol. Below each symbol is that individual's genotype for a microsatellite marker within the *NF1* gene, which provides a basis for identifying which chromosome contains the *NF1* mutation. This information can be used for linkage analysis to diagnose additional family members with a high degree of accuracy. In this family, one can clearly infer that the "5" allele of the polymorphism resides within the *NF1* gene copy containing the pathogenic mutation.

Figure 3. Shown is an autoradiogram from an *NF1* protein truncation test, for segment 3 (spanning exons 19b-29, encoding approximately 660 amino acids, method described previously).[31] The left lane ("normal") shows the pattern from an unaffected individual's leukocyte RNA, where the strongest band near the top of the gel represents the full-length product. The right lane shows the analysis of leukocyte RNA from NF1 patient UF452, which reveals a strong novel lower band (indicated by arrow). This product was found to correspond to a that generated by a nonsense mutation in exon 23-2 (R1362X due to CpG C-to-T mutation), resulting in a protein approximately 40% of the normal length.



A)
1
2
3
4
5
6
226 bp
131 bp
95 bp
B)
1
2
1055 bp
DNA:
Ser
Normal
CATAAGTGAC
Mutation
GGT
Gly
RNA:
Normal
E25
E26
STOP
CGC AGG TGA C
Mutant
E25
E26
68 bp deletion


Normal
UF452


I
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II
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III
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Gene Expression Array Analysis of NF1 Plexiform Neurofibroma Schwann Cell Cultures

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Abstract

Neurofibromatosis 1 (NF1) is a common dominant autosomal disorder occurring in 1 out of 3500 births worldwide. The most characteristic features of NF1 are localized neural crest overgrowths such as neurofibromas, which are benign fibromatous nerve sheath tumors containing primarily Schwann cells. The *NF1* gene encodes the tumor suppressor neurofibromin, a large protein that acts as a ras-GAP in the cytoplasm and may have other functions. Neurofibromin is inactivated in a clonal population of neurofibroma Schwann cells, and thus the two-hit phenomenon at *NF1* appears to be a common first step in tumorigenesis. However, little is known about whether other loci are altered in plexiform neurofibromas, or the spectrum of downstream effects resulting from neurofibromin loss. To better understand these, we used two types of expression arrays to analyze gene expression in a series of human Schwann cell cultures derived from neurofibromas. Overall, the gene expression patterns of the tumor cultures were quite similar to that of normal Schwann cells, although there was some heterogeneity. We have thus far found evidence for aberrant expression in genes involved in apoptosis as well as the extracellular matrix. This supports the notion that Schwann cells derived from plexiform tumors might survive longer than normal Schwann cells and may alter the extracellular matrix, consistent with cell accumulation.

Introduction

Neurofibromatosis type 1 (NF1) is a common dominant autosomal disorder whose most characteristic features are café-au-lait spots and benign fibromatous nerve sheath tumors called neurofibromas. The *NF1* gene is located on chromosome 17q11.2 with a transcript of 11-13 kb containing 60 exons (Viskochil et al. 1993). The only domain thus far with clear biological function lies within exons 20-27a and encodes a RAS-GAP function (Buchberg et al. 1990; Xu et al. 1990). Most mutations in *NF1* lead to predicted truncated proteins either by causing a premature stop via a frame shift or amino acid changes to a stop codon.

Neurofibromin, the *NF1* gene product, contains 2818 amino acids and is expressed at low levels in every cell type, but is most highly expressed in neurons, Schwann cells, and oligodendrocytes (Daston et al. 1992). The GAP domain of this molecule inactivates RAS-p^{21}, and thus neurofibromin is a negative regulator of RAS (Martin et al. 1990). RAS is a proto-oncogenic protein that plays an important role in cell signaling (Wittinghofer 1998) and previous studies have shown that neurofibromas and malignant peripheral nerve sheath tumors (MPNSTs) contain higher levels of activated RAS than non NF1 schwannomas (Feldkamp et al. 1999).

Neurofibromas contain a number of cell types, including Schwann cells, fibroblasts, mast cells, perineurial cells, axons, and vascular elements (Kimura et al. 1974; Riccardi 1992). However, MPNST cells are histologically similar to Schwann cells and perineural cell (Erlandson and Woodruff 1982), and Schwann cells purified from neurofibromas have invasive properties (Muir 1995; Sheela et al. 1990) and cytogenetic abnormalities (Wallace et al. 2000) suggesting that Schwann cells are the clonal, initiating cell type in tumor formation. Also consistent with the two-hit Knudson tumor suppressor model, western blot analysis of Schwann cell cultures derived from dermal and plexiform neurofibromas failed to detect neurofibromin

(Muir et al. 2001; Rutkowski et al. 2000). However, little is known about whether loci other than *NF1* are altered in plexiform neurofibromas or whether loss of neurofibromin is sufficient for tumor formation and/ or progression. Previously, *TP53* LOH has been found in MPNSTs (Legius et al. 1994; Menon et al. 1990) but not in benign neurofibromas (Rasmussen et al. 2000). In addition, overexpression of the platelet-derived growth factor has been found in MPNST-derived Schwann cells (Badache and De Vries 1998) as well as expression of the epidermal growth factor receptor in MPNSTs and benign tumors (DeClue et al. 2000). In another study, the growth factor midkine was found to be upregulated in *Nf1-/-* mouse Schwann cells and expressed in human neurofibromas (Mashour et al. 2001). These studies indicate that mutated Schwann cells proliferate more readily due to aberrant ras activation and expression of growth factors, leading to tumor formation. However, Schwann cell cultures derived from neurofibromas are not excessively proliferative, but do show low tumorigenic potential and are able to delay senescence when compared to normal Schwann cell cultures (Muir et al. 2001).

To further investigate if additional genes and pathways are involved in tumorigenesis of neurofibromas, we constructed a pilot study using cancer-related gene expression arrays to compare Schwann cell cultures derived from plexiform tumors to normal human Schwann cells. We found several genes aberrantly expressed in the tumor cultures compared to normal Schwann cells, and these findings will be discussed.

Methods

Samples.

Tumor and normal (non-NF1) nerve specimens were obtained under IRB approval. Neurofibromin- deficient Schwann cell cultures were established as described in Muir et al. (2001). Total RNA was extracted from cultured cells using the Trizol system (Invitrogen).

Commercial gene expression arrays (Clontech and Affymetrix).

Atlas Human Cancer 1.2 cDNA Expression arrays, which contained cDNA probes from 1200 independent genes, were purchased from Clontech. 20 μg of total RNA were DNase treated, and 4-5μg of total RNA were used for ^{33}P probe generation as outlined by the manufacturer (Clontech). The same normal Schwann cell RNA sample was used for comparison to three tumor samples with each experiment. A total of nine NF1 tumor samples were analyzed using the Clontech system. Hybridization and phosphorimage analysis were performed as outlined in the Atlas protocol (Clontech) using a Storm 480 PhosphorImager (Molecular Dynamics). For the Affymetrix Human Cancer chips, which contained oligonucleotide probes representing 1600 genes, one normal and four tumor Schwann cell culture RNA samples were DNase treated as outlined above and processed by the UF Affymetrix core for fluorescent labeling and hybridization to chips.

Data analysis of array results.

Affymetrix datasets were globally scaled to a target intensity value of 2500 for comparison across all array sets using the Microarray Suite (Affymetrix, Inc.). Comparison analyses were performed between the four tumors and one normal sample, and these Affymetrix data were imported into a custom relational database generated in Microsoft Access 2000 (Microsoft Corporation). Structured Query Language (SQL) was used to perform queries on the dataset.

Clontech cDNA array data were analyzed using the AtlasImage software 1.01 package (Clontech) and each membrane was globally normalized. The Atlas array analysis selected for genes that showed intensity values 2X above the background to minimize false positives. The data were converted into fold changes, imported into a relational database and similar queries as above were performed. A third relational database was constructed where both sets of data were imported and queried simultaneously. This screen resulted in a list of genes whose tumor mRNA levels appeared to deviate from normal Schwann cell values most dramatically and in the most tumor samples. Heirarchical clustering was attempted, with the Affymetrix data (Atlas data not being amenable), but the sample number was too low for meaningful results.

Light Cycler analysis.

1 μg of DNase treated total RNA from normal and tumor Schwann cell cultures was reversed transcribed using random hexamer primers and Superscript II (Invitrogen) in a 20 μl volume. 1 μl of diluted (1:5) cDNA was used as template in a LightCycler rapid real-time thermal cycler system (Roche) as follows: 5 second at 94^{0}C, 5 second at primer-specific annealing temperature, 30 seconds at 72^{0}C for 40 cycles in a 10 μl volume using the Fast Start DNA Master SYBR Green I mix (Roche). RT-PCR primer sequences for a set of 11 chosen genes are shown in Table 1. Fluorescent PCR product detection was carried out at the end of each 72^{0}C extension step by the LightCycler. A melting curve analysis was generated for each primer pair to confirm amplification specificity. Recovered (post-PCR) samples were ethidium bromide visualized on an 8% native polyacrylamide gel to confirm specificity. *HPRT* expression levels were used for normalization. A standard curve was generated by amplifying serial dilutions (10^2-10^8 molecules) of a known quantity of purified PCR product specific for each gene. Each data point was transformed to ^{10}log format and the least-squares regression line (y = ax + b) was used to

determine values for each sample. Calculated values were converted into molecules to obtain fold changes of these gene mRNA levels in tumor versus normal Schwann cells.

Results

Commercial Human Cancer cDNA Expression Arrays.

We analyzed total RNA from one normal Schwann cell culture and nine plexiform Schwann cell cultures using the Atlas Human Cancer 1.2 cDNA Expression Arrays, and four tumor Schwann cell samples and the same normal sample using the Affymetrix Human Cancer chips, which included three plexiform cultures analyzed on the Atlas arrays. Statistical queries were designed to show only genes that were differentially expressed in at least 3 samples for either array type, and Table 2 shows the results from the Atlas arrays. The minimum, maximum, mean, and standard deviation values for each gene were calculated based on tumor/ normal fold change. The same calculations were performed on the Affymetrix data and the results are shown in Table 3. A total of 11 genes were detected under these criteria. *TIMP3*, *P75*, *TSP2*, and Integrin beta 8 were represented on both arrays. *NOTCH4* is only present on the Atlas array, while *GFRA2*, *HCG*, and *hIGFBP* are only on the Affymetrix chip. *TRAIL*, *MT1*, and *HYAL1* are present on both types of arrays, but appeared only differentially expressed on the Affymetrix system, with the exception of *MT1*, which was positive in two Atlas-queried samples.

Real Time PCR Analysis.

To confirm differential expression of the 11 candidate genes, we employed real time PCR analysis. The *HPRT* housekeeping gene was used for normalization based on similar expression levels seen in all the samples, and precedent in the literature (Steuerwald et al. 2000). We tested all the samples from the Affymetrix chip analysis and eight out of nine plexiform Schwann cell

cultures from the Atlas arrays. For quantification of gene expression of normal versus tumor samples, we created a standard curve to calculate number of molecules present in each sample for each gene, which were converted to fold changes (tumor/ normal). The results are shown in Table 4. The genes were all found to be consistently differentially expressed, except for *NOTCH4* and *hIGFBP1*. While *NOTCH4* was found to be upregulated in the Atlas array analysis, the majority of samples showed downregulation of this gene by real time PCR. The opposite situation was found for *hIGFBP1*, in which most samples showed upregulation with real time PCR. Thus, overall most of the genes had consistent expression changes in the same direction, although the exact fold changes vary depending on the sensitivity of method used. There is no basis for prediction of functional effect based on transcript level changes, since for some genes/ proteins a small change could have dramatic protein-level effect, and vice versa. Thus, the most important factor is verifiable up- or down-regulated expression difference (not precise fold-change), which we have shown with a highly sensitive confirmatory method.

Discussion

The genes confirmed to be altered in expression most consistently fall into two major pathways that could contribute to tumorigenesis: apoptosis and extracellular matrix. Genes regulating the cell cycle, or growth factors that would give Schwann cells advantages in proliferation, did not appear to be significantly altered. Instead, our findings support the theory that NF1-null Schwann cells are able to delay senescence by downregulation of apoptosis-regulators such as *TRAIL* and *P75*. TRAIL is a type II integral membrane protein that induces apoptosis in a number of tumor cells (Pitti et al. 1996; Wiley et al. 1995) by activating a caspase cascade (Sprick et al. 2000; Bodmer et al. 2000). P75 is highly expressed in Schwann cells

(Lemke and Chao 1988) and is related to the tumor necrosis factor receptor and Fas (Meakin and Shooter 1992). NGF (nerve growth factor) induced apoptosis has been shown to be mediated by p75 (Soilu-Hanninen et al. 1999) activating the c-Jun N-terminal kinase (JNK) pathway (Yoon et al. 1998). A small reduction in apoptosis rate is consistent with the growth of a slow growing tumor. However, since tumor derived Schwann cells will eventually undergo apoptosis, this indicates that other apoptosis signaling pathways are still functional.

In addition, changes in expression of genes involved in the extracellular matrix such as *TIMP3, MT1*, *TSP-2*, and *HYAL-1* could contribute to changes that would favor tumor growth by creating a more favorable environment for tumorigenic Schwann cells. Matrix metalloproteinases (MMPs) are endopeptidases known to break down extracellular components, and inhibitors of MMPs (TIMPs) function to counteract the effects of MMPs to maintain a balance between deposition and degradation in the extracellular matrix (ECM). TIMPs have a variety of functions such as inhibiting MMPs, inducing apoptosis, and inhibiting tumor cell invasion and metastasis. TIMP*3* binds tightly to the ECM and overexpression of *TIMP3* has been shown to reduce attachment of transformed cells leading to apoptosis (Ahonen et al. 1998) and to inhibit TNFalpha converting enzyme (Amour et al. 1998), which in turn induces apoptosis by stabilization of the TNFalpha receptor on the cell surface (Smith et al. 1997). Thrombospondin 2 (*TSP-2*) is an extracellular matrix glycoprotein that inhibits angiogenesis, plays a role in controlling tumor growth by contributing to the host anti-tumor defense mechanism (Hawighorst et al. 2001), and supports cell attachment and cell-cell and cell-matrix interactions (Bornstein et al. 2000; Yang et al. 2000). Therefore, *TSP2* expression could aid in the suppression of apoptosis since anchorage dependent cells must adhere to the matrix to survive in neurofibromas. Hyaluronidase 1 (*HYAL1*) breaks down hyaluronan (Frost et al. 1997), which is a major

glycosaminoglycan of the extracellular matrix that functions in matrix assembly, cell proliferation, migration, differentiation, and as a signaling molecule (Lee and Spicer 2000). *HYAL1* was found inactivated in head and neck squamous cell carcinomas (Frost et al. 2000), yet overexpression of *HYAL1* seems to enhance tumor formation in prostate cancer (Patel et al. 2002). Since expression of *HYAL1* has been implicated in aiding tumor progression as well as reducing tumor growth, its exact function might be cell specific. Of interest, the first four samples in Table 4. are from males, and show consistent patterens (and the normal control was from a male). Thus, gender may explain some heterogeneity between tumor results.

Our study does not address whether neurofibromin deficiency alone is sufficient for tumor formation or if other genes are involved as well. However, since we did find alterations in gene expression in the tumor cultures, most likely other genes are also involved in the tumorigenesis of plexiform neurofibromas. Since the genes identified in this study are involved either directly or indirectly in apoptosis and the ECM, changes in these pathways in plexiform Schwann cells could be contributing to tumor progression. In addition, the alteration in the pathways identified in this study support the growth characteristics typically found in those tumors and tumor derived cultures. Since neurofibromas contain a mixture of cell types, array-based analysis was most feasible using RNA from characterized Schwann cell enriched cultures rather than from primary tumor samples. Since normal Schwann cell cultures were used for comparison, this minimizes the risk that gene expression alterations are artifacts caused by culture effects. These findings may shed new light on NF1 tumorigenesis and aid in the development of new therapeutic treatments for NF1.

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TABLE 1. PRIMER SEQUENCES FOR REAL TIME PCR ANALYSIS.

Gene	Primer Sequences
GFRA2	5'-ATGCCAATTGTCGAGCCTCC-3'
	5'-GCAGGTGGTGATGACACTGGTCC-3'
HYAL1	5'-GTGCTGCCCTATGTCCAGAT-3'
	5'-CAGGGTTAAGGAGGAGGAGG-3'
HCG	5'-GGGCATCCAAGGAGATGCTT-3'
	5'-TCATCACAGGTCAAGGGGTG-3'
NOTCH4	5'-CACGTGAACCCATGTGAGTC-3'
	5'-TTGAGCAGTTCTGTCCATCG-3'
MT-1 (MMP14)	5'-GGAGACAAGCATTGGGTGTT-3'
	5'-GGTAGCCCGGTTCTACCTTC-3'
TRAIL	5'-ACCAACGAGCTGAAGCAGAT-3'
	5'-TCCTTGATGATTCCCAGGAG-3'
P75	5'-CTGCAAGCAGAACAAGCAAG-3'
	5'-CTGCACAGACTCTCCACGAG-3'
TSP2	5'-GCAACATCAACCGCAAGAC-3'
	5'-AAGCAAACCCCTGAAGTGACT-3'
TIMP3	5'-GCCTTCTGCAACTCCGACATC-3'
	5'-CGTGTACATCTTGCCATCATA-3'
INTEGRIN ß8	5'-ATCTGTCCAGATGGGTCCAG-3'
	5'-GCACACAGATTTCCATGGTG-3'
IGFBP1	5'-GATCATTCCATCCTTTGGGA-3'
	5'-GAGACCCAGGGATCCTCTTC-3'
HPRT	5'-CCTGCTGGATTACATTAAAGCACTG-3'
	5'-CCTGAAGTACTCATTATAGTCAAGG-3'

TABLE 2. ATLAS HUMAN CANCER 1.2 cDNA ARRAY DIFFERENTIALLY EXPRESSED GENES.

Gene	UF572	UF609	UF632	UF440	UF554	UF327	UF550	UF362	UF378	MIN	MAX	MEAN	STDEV
TIMP3	--	--	--	--	+	+	--	ND	ND	-2.62	1.08	-0.88	1.29
NOTCH4	+	+	+	--	+	+	--	+	+	-1.83	1.86	0.62	1.22
P75	--	ND	--	--	--	ND	ND	ND	ND	-37	-1.03	-10.2	12.2
TSP2	ND	ND	+	+	+	ND	+	ND	ND	1.34	1.92	1.62	0.87
INTEGRIN β8	--	--	+	--	+	+	+	ND	+	-1.46	1.74	0.33	1.25

-- = Downregulated genes compared to normal Schwann cell culture
+ = Upregulated genes compared to normal Schwann cell culture
ND = Sample did not show differential expression for that gene

TABLE 3. AFFYMETRIX ARRAY DIFFERENTIAL EXPRESSED GENES.

Gene	UF378	UF440	UF572	UF511	MIN	MAX	MEAN	STDEV
TIMP3	--	--	--	--	-4.3	-2.1	-2.75	1.04
TIMP3	--	--	--	--	-4.7	-2.1	-2.8	1.26
TRAIL	--	--	--	--	-6	-4.1	-5.35	0.9
P75	--	--	ND	ND	-17	-2.9	-9.95	8.13
TSP2	+	+	+	--	-4.5	16.7	5.8	8.69
TSP2	+	+	+	ND	6.4	17.3	11.9	7.43
MT1	--	--	--	--	-8.7	-2.3	-5.05	3.26
GFRA2	--	--	ND	--	-7.3	-3.8	-5.13	3
HCG	--	--	--	ND	-9	-2.3	-5.27	3.84
HIGFBP1	--	--	--	--	-4.4	-2.3	-3.17	1.82
HYAL1	--	--	ND	--	-7.4	-3.1	-5.37	3.21
INTEGRIN β8	--	--	--	ND	-7.3	-3.3	-5.87	3.45

-- = Downregulated genes compared to normal Schwann cell culture
+ = Upregulated genes compared to normal Schwann cell culture
ND = Sample did not show differential expression for that gene

TABLE 4. REAL TIME PCR ANALYSIS OF DIFFERENTIAL EXPRESSED GENES.

Gene	UF327	UF378	UF362	UF609	UF550	UF511	UF440	UF572	UF554
TIMP3	--	--	--	--	--	--	--	--	--
NOTCH	--	--	--	--	--	--	+	+	+
TRAIL	--	--	--	--	--	--	--	--	--
P75	--	--	--	--	--	+	--	+	+
TSP2	+	+	+	+	+	--	+	+	--
MT1	--	--	--	--	--	--	--	--	+
GFRA2	--	--	--	--	--	--	--	--	+
HCG	--	--	--	--	--	--	--	+	+
HYAL1	--	--	--	--	--	--	--	--	+
hIGFBP1	+	+	+	+	--	--	--	+	+
INTEGRIN β8	--	--	--	--	--	--	--	--	--

-- = Downregulated genes compared to normal Schwann cell culture
+ = Upregulated genes compared to normal Schwann cell culture